TRAFFICKING AND ACTIVITY DEPENDENT FUNCTION OF VESICULAR TRANSPORTERS

by

Lesley Anne Colgan

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This dissertation was presented

by

Lesley Anne Colgan

It was defended on

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and approved by

Thomas Martin, Professor, Dept. of Biochemistry, University of Wisconsin Madison

Susan Amara, Detre Professor and Chair, Dept. of Neurobiology, University of Pittsburgh

John Horn, Professor, Dept. of Neurobiology, University of Pittsburgh

Adrian Michael, Associate Professor, Dept. of Chemistry, University of Pittsburgh

Gonzalo Torres, Assistant Professor, Dept. of Neurobiology, University of Pittsburgh

Yongjian Liu, Research Assistant Professor, Dept. of Neurobiology, University of Pittsburgh

Edwin Levitan, Professor, Dept. of Pharmacology and Chemical Biology, University of Pittsburgh

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Lesley Anne Colgan, PhD

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Vesicular neurotransmitter transporters (VNTs) are a small family of proteins responsible for packaging neurotransmitter into secretory vesicles. Their presence and function are required for regulated secretion from neuronal and neuroendocrine cells. During both the biogenesis and the activity-dependent recycling of secretory vesicles, VNTs undergo trafficking that can determine the quality, quantity, and location of packaged neurotransmitter. Thus understanding the signals and mechanisms of VNT trafficking is essential to understanding the regulation of neurotransmission.

Here, the synaptic vesicle specific trafficking of Vesicular Acetylcholine Transporter (VAChT) is investigated. A dileucine containing targeting motif, with dual properties for internalization and synaptic vesicle targeting, is identified in the C-terminus of VAChT. Chimeras between this motif and an unrelated plasma membrane protein localize to synaptic-vesicle-like vesicles in a neuroendocrine cell line. The specificity and generalization of this motif is assessed. Next, sorting nexin 5 (SNX5), implicated in the regulation of membrane traffic, is identified as a novel regulator of VAChT targeting to synaptic vesicles. Disruption of SNX5 function leads to a decrease in VAChT-directed synaptic vesicle targeting and a

concomitant increase in targeting to large dense core vesicles. This shift between secretory granules suggests an important mechanism of VNT regulation with the potential to shape properties of neurotransmission.

In order to understand the physiologic importance of VNT regulation, vesicular transport and its influence on activity-dependent release must be assessed in living neurons. However, this has not been possible. Therefore, a live cell assay was established to measure vesicular transport and its contributions to release in brain slice. Using a pH sensitive, fluorescent serotonin analog visualized by two-photon microscopy, activity dependent somatic release and vesicular monoamine transporter (VMAT) activity were measured in the dorsal raphe nucleus. Interestingly, while a portion of monoamine packaged at rest was held in reserve, monoamine packaged during stimulation was released efficiently. The work presented in this thesis provides a greater understanding of VNT trafficking and activity-dependent function. Furthermore, it provides the foundation for the comprehensive study of the active role of VNTs in shaping the properties of neurotransmission.

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PREFACE

Acknowledgements

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List of Abbreviations

5-HT- 5- hydroxytryptamine, serotonin

Ach- acetylcholine

DA- dopamine

DAT- dopamine transporter

DHT- 5,7-dihydroxytryptamine

E- epinephrine

Glu- glutamate

ISG- immature secretory granule

LDCV- large dense core vesicle

NE- norepinephrine

NT- neurotransmitter

PMT- plasma membrane transporter

SDCV- small dense core vesicles

SGII- secretogranin II

SV- synaptic vesicle

SVLV- synaptic vesicle like vesicle

SVTM- synaptic vesicle targeting motif

Syn- synaptophysin

v-ATPase- vacuolar type ATPase

VAT- vesicular amine transporter

VAChT- vesicular acetylcholine transporter

VGluT- vesicular glutamate transporter

VIAAT-vesicular inhibitory amino acid

VMAT- vesicular monoamine transporter

VNT- vesicular neurotransmitter transporter

VNUT- vesicular nucleotide transporter

1.0 INTRODUCTION

1.1 VESICULAR NEUROTRANSMITTER TRANSPORTERS AND NEUROTRANSMISSION

Vesicular neurotransmitter transporters (VNTs) are required for regulated secretion from neuronal and neuroendocrine cells. Described as the 'gate keeper' (Eiden, 2000) for secretory vesicles, this small family of proteins is responsible for the vesicular concentration and packaging of neurotransmitters. Classically thought to be an invariant passageway, current evidence described below suggests that VNTs determine the quality, quantity, and location of the neurotransmitter packaged and consequently the parameters of neuronal signaling. Thus, VNTs are not only required for the maintenance of neurotransmission, but likely play a more active role in its regulation.

1.1.1 Neurotransmitter and Secretory Vesicle Cycles

The maintenance of neurotransmission is reliant on concurrent cycles of neurotransmitter and secretory vesicles through packaging, release, and recycling (Figure 1). The convergence of these cycles is the packaging of transmitter into secretory vesicles mediated by the VNT family. This active transport highly concentrates transmitter, while sequestering it from degradative enzymes. Furthermore, it provides efficient storage of the chemical prior to stimulation-induced release. Upon the generation of a nerve impulse, local calcium entry triggers the fusion of

secretory vesicles with the cell membrane to release their neurotransmitter content. This can occur via complete fusion, in which the vesicles collapse into the cellular plasma membrane releasing their content, or via 'kiss and run', in which a transient fusion pore connecting the vesicle to the plasma membrane opens, allowing the release of vesicular content while maintaining vesicle identity. Once in the extracellular milieu, neurotransmitter interacts with receptors in nearby cells to impart a molecular response, thereby mediating 'information transfer' from the releasing neuron to neighboring cells. Signal termination in most cases is mediated by reuptake of the released transmitter by specific plasma membrane transporters (PMT) into the releasing neuron and/or nearby cells. PMT mediated uptake not only removes transmitter from the extracellular space, but also allows for transmitter recycling for future release. For acetylcholine (Ach), signal termination is mediated by the degradation of extracellular transmitter. However, the metabolite choline undergoes specific reuptake for reuse in cholinergic transmission. In addition to transmitter, secretory vesicles and associated proteins involved in regulated release, including VNTs, are also recycled to maintain synaptic efficacy. During 'kiss and run' modes of release, reformation of secretory vesicles is through direct closing of the fusion pore. However, during full fusion transmission, the lipid and protein content of secretory vesicles must be sorted and retrieved through a slower, clathrin-dependent endocytosis. The concomitant recycling of transmitter and secretory vesicles allow for neurotransmitter to be efficiently repackaged by the VNT family into secretory vesicles to support further neurotransmission.

Disruption of individual steps in either the neurotransmitter or vesicle cycles leads to alterations in neurotransmission. For example, genetic disruption of the Drosophila isoform of dynamin, which is involved in vesicle recycling, leads to rapid paralysis as sustained

neurotransmission fails. Ultrastructure analysis of synapses show a marked decrease or total depletion of synaptic vesicles from terminals, consistent with a defect in vesicular recycling (Poodry and Edgar, 1979). Alternatively, disruption of the neurotransmitter cycle through knockout of the plasma membrane transporter for dopamine (DAT) leads to neurotransmission deficits and behavioral alterations. Consistent with a role of DAT in signal termination and recycling of transmitter, knockout animals show a 300-fold lengthening of the dopaminergic signal and a 20 fold decrease in dopaminergic vesicular stores (Gainetdinov et al., 1998).

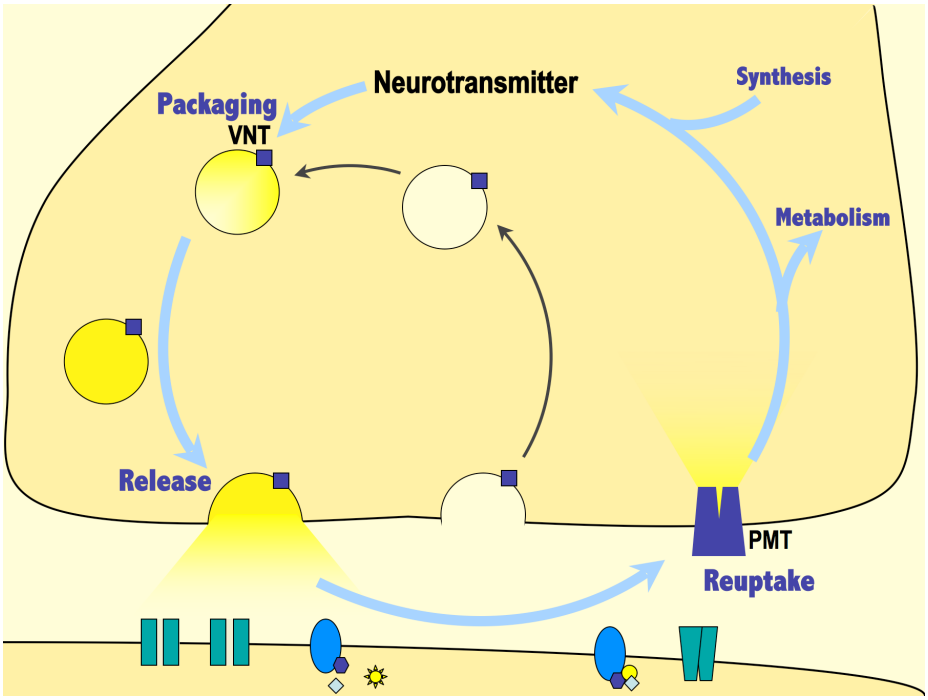

Figure 1. Neurotransmitter and Vesicle Cycles

Concurrent neurotransmitter and vesicle cycles maintain synaptic efficacy. The neurotransmitter cycle (blue arrows) consists of NT packaging mediated by the VNT family, release of NT extracellularly to impart a signal, and the reuptake through PMTs. The synaptic vesicle cycle (black arrow) consists of vesicle filling by the VNT family, release upon stimulation, and recycling through endocytosis. Recycled transmitter is packaged into reformed vesicles to support further release.

The highly regulated sequences of events in both the neurotransmitter and vesicle cycles are essential for neurotransmission. Understanding each of these steps will provide a better understanding of the normal maintenance of transmission as well as perturbations that underlie neuronal dysfunction in disease states. This thesis will focus on the role of VNTs in the context of neurotransmission. While known to be essential for neurotransmission, until recently the potential of VNTs to regulate neuronal signaling has been underappreciated. Thus, many questions remain about their regulation. Below I will describe the current understanding of VNT function, including a brief history of their discovery and characterization, the potential for the physiologic and pathologic regulation of VNTs and subsequent consequences for neurotransmission, and finally the potential mechanisms of mediating this regulation through the trafficking of VNTs to secretory vesicles. I will end with a brief discussion on current techniques to assess vesicular packaging and the need for an intact live cell assay.

1.1.2 Vesicular Neurotransmitter Transporter (VNT) Function

The packaging of neurotransmitters into secretory granules is mediated by a family of proteins, the vesicular neurotransmitter transporters (VNTs). Three genetic sub-families of VNTs have been characterized: the inhibitory amino acid transporter family (VIAAT; SLC32) which transports the classically inhibitory transmitters GABA and glycine; a vesicular glutamate transporter family (VGluT; SLC17) consisting of three transporters responsible for the transport of glutamate, $VGluT_1$, $VGluT_2$, $VGluT_3$ and a newly identified vesicular transporter for ATP, VNUT; and finally a vesicular amine transporter family (VAT; SLC18) consisting of transporters for acetylcholine and the biogenic amines. This family consists of the vesicular acetylcholine transporter (VAChT), and the vesicular monoamine transporters ($VMAT_1$ and $VMAT_2$), which

package dopamine (DA), serotonin (5-HT), norepinephrine (NE), epinephrine (E), and histamine. The VAT family is the most widely studied of the vesicular transporters due to the central role of the cholinergic and aminergic transmitter systems in mental health and neurodegenerative disorders, and as such, it will be the focus of this thesis.

1.1.3 VNT Discovery and Characterization

The discovery of VNTs stemmed from the identification of secretory granules as the storage sites for neurotransmitters and the subsequent isolation of these granules. The abundance of chromaffin granules in the adrenal medulla and the high concentrations of monoamine stored in them provided an ideal model system for studying neurotransmitter transport and storage. It is not surprising, therefore, that the earliest isolation and characterization of secretory vesicles was from bovine adrenal medulla in work by Hillarp and colleagues during the mid to late 1950's (Hillarp, 1958a, b). These studies used density gradient centrifugation to isolate secretory granules and characterize their neurotransmitter content as well associated ATPase activity. This work, along with the transformational work of Katz and colleagues on quantal acetylcholine release at the neuromuscular junction (Del Castillo and Katz, 1954) and the advancements in electron microscopy, which provided the first visual images of uniformly sized membrane compartments within the nerve terminal (Robertson, 1956), helped to solidify the concept of vesicular transmitter release (Del Castillo and Katz, 1956). The early characterization of VNTs, and in particular, VATs, was aided greatly by the use of specific drugs that disrupted storage of neurotransmitters in secretory vesicles. Particularly the use of reserpine which inhibits monoamine transport (Hillarp, 1960; Jonsson and Sachs, 1969), and later vesicamol which blocks cholinergic transport (Marshall, 1970). Interestingly, reserpine, a widely used treatment

for hypertension at the time, showed severe side-effects of depressive symptoms in humans, an early indication of the centrality of monoamine transport to mental health (Freis, 1954).

1.1.4 Biophysical Properties of Vesicular Transport

In the 70's and 80's considerable work was done on the biophysical properties of transport. Studies examining the vesicular concentrations of transmitters made it clear that vesicular transport was an active process requiring energy to package neurotransmitters against their concentration gradient. By 1979, adrenergic transport function had been reconstituted after solubilization of chromaffin granules and transfer of isolated protein to liposomes. Transport function was activated with the addition of an artificial pH gradient. As with endogenous transport, the reconstituted transport was inhibited by reserpine and required ATP as an energy source (Maron et al., 1979).

The requirement of ATP in the accumulation and storage of secretory vesicle content suggested that ATP hydrolysis might provide the transport energy for the movement of transmitter into the vesicle. Studies in the early 80's identified the dependence of the transport on a proton gradient, in which energy was derived from the transport of H^+ ions down their electrochemical gradient (Anderson et al., 1982; Toll and Howard, 1980). The Mg^{+2}-dependent vacuolar-type H^+-ATPase (v-ATPase), the same protein known to mediate the acidification of lysosomes, was soon identified as the source of the proton gradient (Cidon and Sihra, 1989). The v-ATPase hydrolyzes ATP to translocate protons into secretory vesicles generating an electrochemical gradient that can be used to drive the active packaging of neurotransmitter. A measurable granule chemical potential (ΔpH~ -1.4) and electrical potential ($\Delta\Psi$ ~ +39 mV) is harnessed by the VNT family to exchange movement of neurotransmitter into the vesicle with

protons out of the vesicle. For the VATs the stoichiometry of this exchange is a single molecule of Ach^+ or $monoamine^+$ for every 2 H^+ transported out of the vesicle (Knoth et al., 1981). The charge exchange of this stoichiometry (net movement of one positive charge out of the vesicle) compared to the chemical exchange (the net movement of two hydrogen ions out of the vesicle) suggests that the VAT family relies more on the chemical potential than the electrical potential to mediate transport (Figure 2). Thus, perturbations of the chemical gradient are more effective in disrupting storage of monoamines or Ach than alteration of the vesicular electrical gradient.

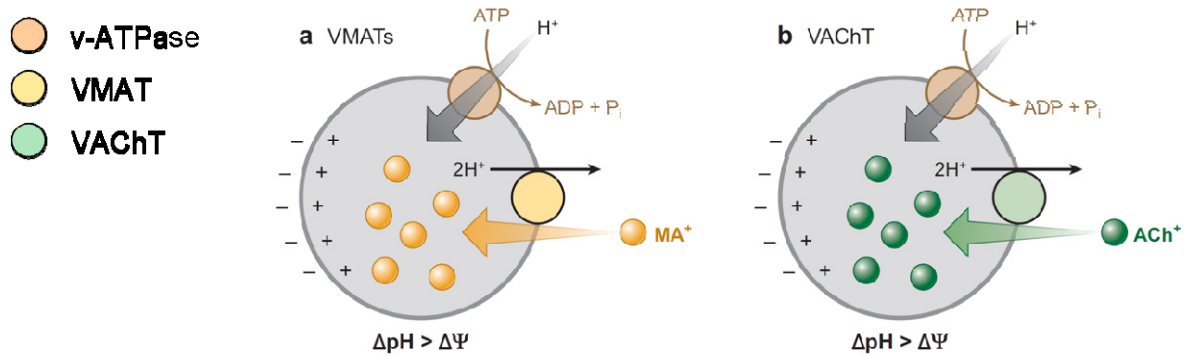

Figure 2. Vesicular transport relies on a H^+ electrochemical gradient.

The vesicular transporters mediate active packaging of transmitter by coupling neurotransmitter translocation with the running down of a H^+ electrochemical gradient. A vacuolar-type H^+-ATPase (V-ATPase) continually generates a H^+ gradient across the vesicle membrane by hydrolyzing ATP and transporting H^+ into secretory granules. This generates a chemical gradient (Δ pH) as well as an electrical gradient ($\Delta \Psi$) that are harnessed for active transport of neurotransmitter into the vesicle. Depending on the charge of the neurotransmitter substrate, vesicular transporters rely to different extents on the two components. VMATs and VAChT transport their positively charged substrates in exchange for two H^+, and hence rely primarily on Δ pH. (modified from Chaudhry et al., 2008).

The generation of the electrochemical gradient described above predicts energy to support a concentration gradient of vesicular NT on the order of ~10^4 relative to cytoplasmic NT. While vesicular concentration gradients vary greatly *in-vivo,* monoamines gradients have been reported to reach concentrations upwards of 10^5. Thus, predicted concentrations of NT are an order of magnitude less than can be measured *in vivo*. One explanation of this difference is that monoamines form insoluble aggregates through intermolecular interactions in vesicles. This reduces their 'effective' concentration and allows for the further concentration of transmitter. Surprisingly, concentration gradients of Ach are often lower than that predicted by the gradient potential. Thus, VAChT transport seems to be less efficient than VMAT transport. Furthermore, the affinity of VAChT for Ach (mM range) and its transport rate (~1/s) is lower than those of VMAT (km ~ μM range; turnover ~10/s). In addition to H^+ as a counterion, a role for chloride in vesicular transport has been described. The transport of the negatively charged chloride ion into the vesicle would allow dissipation of the proton electric gradient and thus further increase the concentration of H^+ inside the vesicle. The role for chloride transport is not well understood, but is thought to be particularly relevant for VGluT function (Moriyama and Yamamoto, 1995).

1.1.5 Cloning and Molecular Characterization of VNTs

In the early 90's, the vesicular transporters were molecularly characterized through their cloning and sequencing. The first vesicular transporters cloned were the VMAT family, independently cloned by two groups using different strategies. Liu and colleagues assayed resistance to the active neurotoxic metabolite MPP^+, implicated in a Parkinsonian-like disease phenotype. Chromaffin cells and the neuroendocrine PC12 cell line showed resistance to MPP^+ toxicity, however a non-aminergic cell line, Chinese hamster ovary (CHO) cells, showed sensitivity.

Thus, to investigate the protein responsible for MPP^+ resistance, a cDNA library from PC12 cells was transformed into CHO cells and clones were selected for viability upon MPP^+ exposure. Selection and subsequent screening of clones resistant to toxicity led to the identification of the protein that conferred resistance to MPP+, later understood to mediate resistance by the sequestering of the toxin in secretory vesicles. The expression of the identified clone shifted dopamine from a cytoplasmic to a punctate distribution. These characteristics were found to be reserpine dependent, suggesting the identification of the protein as the putative vesicular transporter. Finally, the putative transporter was shown to transport dopamine in a reconstituted system with comparable biophysical and pharmacologic properties as previously characterized (Liu et al., 1992a; Liu et al., 1992b). In addition to the isolated gene product, vesicular monoamine transporter 1 ($VMAT_1$), the group identified through homology an additional closely related family member ($VMAT_2$). Around the same time, $VMAT_2$ was independently cloned and verified by another group by assaying serotonin uptake. DNA derived from RBL cells, which transport and store serotonin in secretory granules, was transfected into a non-aminergic host cell and clones demonstrating high levels of serotonin uptake were selected (Erickson et al., 1992). The two independently verified VMAT proteins share high sequence homology, encoding polypeptides with twelve transmembrane domains, a large luminal loop, and cytoplasmic N and C termini domains (Liu et al., 1992a). Sequence differences between the two proteins were found primarily in the N and C termini domains and the luminal loop. Distribution analysis revealed complementary expression patterns with $VMAT_1$ expressed primarily in the peripheral nervous system and $VMAT_2$ in the brain (Peter et al., 1995). Finally, functional analysis of the two proteins showed similar transport of all of the biogenic amines with the exception of histamine, for which $VMAT_2$ has higher affinity (Liu et al., 1996).

The cloning of VAChT soon followed (Alfonso et al., 1993; Erickson et al., 1994; Roghani et al., 1994; Varoqui et al., 1994). It was first cloned by analysis of a previously characterized mutation in *c. elegans*, UNC-17, that showed paralysis and resistance to aldicarb, and inhibitor of synaptic acetylcholine metabolism. The mutated gene in UNC-17 was cloned and sequenced and showed high homology (~ 40% identity and 65% similarity) to the VMAT family and a similar predicted structure of twelve transmembrane domains and cytosolic N and C termini tails. Together with the functional analysis of the mutant, UNC-17 was identified as a putative vesicular acetylcholine transporter. Since the cloning of the VAT family, the transporters for glutamate (VGluT 1-3), and GABA/ glycine (VIAAT) have been identified and characterized (Bellocchio et al., 2000; McIntire et al., 1997; Takamori et al., 2000). Recently, the vesicular nucleotide transporter (VNUT), a member of the VGluT gene family, has been cloned and identified as the vesicular ATP transporter (Sawada et al., 2008).

1.1.6 Genetic Alteration and Knockdown Studies

The vesicular hypothesis of transmission, suggested that vesicular transporter function was required for neurotransmission. In fact, the presumed centrality of this family to proper neuronal function led to the widespread use of markers of VNT density as indicators of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease. The essential role of these proteins to neurotransmission was confirmed directly by the genetic disruption of the vesicular transporters. The mammalian knockout of $VMAT_2$ was completed in the late 90's by several groups of investigators who found the knockout to be perinatal lethal. Knockout mice showed drastically reduced monoamine levels and disruption of vesicular release (Fon et al., 1997; Takahashi et al., 1997; Wang et al., 1997). Null mutants of the VAChT gene in *c. elegans* were

also lethal (Alfonso et al., 1993). Interestingly, cellular analysis of drug and knockout studies revealed that while disruption of transporter function blocked the loading of synaptic vesicles with transmitter and thus neurotransmitter signaling, the biogenesis of secretory vesicles and the vesicle cycle were not disrupted. Thus empty vesicles were found to cycle normally, suggesting that the vesicle cycle is 'blind' to vesicular neurotransmitter content (Croft et al., 2005; Parsons et al., 1999). This finding indicates that the amount of transmitter release is determined by vesicular transport, including the expression and localization of transporters to vesicles, rather than a checkpoint for the selective release of filled vesicles. This, together with other emerging evidence discussed below, suggests an active role of VNTs in shaping the properties of synaptic transmission. Thus in the last decade focus in the field has shifted toward understanding the potential regulation of the VNTs.

1.2 REGULATION OF VNTS

1.2.1 Quantal Size

Quantal size is defined as the neuronal electrical response to the release of a single packet, or quantum, of neurotransmitter. The term was introduced in studies by Bernard Katz on the postsynaptic responses to spontaneous and stimulated release at the neuromuscular junction (For review see Augustine and Kasai, 2007). As implied by the name, quanta were widely considered invariant; thus, when changes in quantal size began to be appreciated the mechanisms were believed to be postsynaptic in nature. The prevalent dogma was that the amount of neurotransmitter released per synaptic vesicle was fixed and that only changes in the receptor

response to that transmitter could underlie changes in quantal size. This was further supported by an assumption that the amount of transmitter released by a single synaptic vesicle would saturate postsynaptic receptors (Frerking and Wilson, 1996). If this were the case, any increase in the amount of transmitter released per vesicle would have no physiologic effect on signal transmission. However, close examination of the spontaneous release of single vesicles (mPSPs) or stimulated release (PSPs) has shown that under physiologic conditions significant variations in quantal size exist. More recent studies have determined that even at the high-affinity NMDA glutamate receptor this variation is present and due to alterations in the amount of neurotransmitter released (Liu et al., 1999; Mainen et al., 1999; McAllister and Stevens, 2000). Thus, at least most of the time receptors are not saturated and the amount of transmitter released per vesicle shows significant variation. At aminergic terminals the majority of released transmitter interacts with slower, metabotropic receptors and is paracrine in nature, often not confined to highly local synapses. In this case, alterations in quantal size are likely to have a large effect on signal transduction, both by altering the number of receptors activated as well as the duration of their activation. Thus, understanding presynaptic changes in quantal size is relevant to understanding neurotransmission.

1.2.2 Presynaptic Regulation of Quantal Size

In addition to the *in-vivo* variation of quantal size at many synapses, a growing body of work suggests that these presynaptic changes are regulated. Perhaps the most physiologic of these regulations is seen *in-vivo* with the movement of Drosophila from a plate containing food to one without. Concurrent with an increase in foraging and crawl speed, an increase in quantal size is

seen within 35 minutes. This increase is due to changes in presynaptic mechanisms, namely the amount of transmitter stored in synaptic vesicles (Steinert et al., 2006).

Moreover, experimental paradigms demonstrate activity-dependent modulation of quantal size by presynaptic mechanisms. For example, disruption of neural activity of cholinergic neurons leads to a concomitant increase in quantal size (Van der Kloot and Molgo, 1994; Wang et al., 2005). On the other hand, high frequency stimulation of the neuromuscular junction reduced quantal size, independent of postsynaptic changes (Doherty et al., 1984). The molecular mechanisms that mediate these changes are not well understood, although broad perturbation of signaling molecules, such as activation of protein kinase A (PKA) and inhibition of protein kinase C have been shown to increase and decrease quantal size respectively, suggesting that these changes are regulated (Staal et al., 2008; Van der Kloot and Branisteanu, 1992).

Presynaptic alterations in quantal size reflect changes in the amount of NT released from a single synaptic vesicle. This could be modulated by one or both of the following mechanisms: (1) changing the amount of NT contained in the vesicle or (2) the amount of NT released from the vesicle upon exocytosis. In the case of the latter, the amount of NT released per vesicle could be modulated through regulation of the size or stability of the fusion pore during 'kiss and run' modes of release (Jackson and Chapman, 2008). However, studies suggest that for small (non-peptidergic) transmitters, the fast rise times of spontaneous release events ($< 100\ \mu s$) and the size of secretory vesicles demonstrate that even for unstable fusion pores all NT would be released (Klyachko and Jackson, 2002). Thus, changes in fusion pore stability would be unlikely to influence quantal size. During full fusion modes of release, the content of each vesicle is completely released, eliminating this possibility for this type of variation.

Thus, presynaptic alterations of quantal size most likely reflect changes in the amount of neurotransmitter stored in secretory vesicles. Biophysical understanding of vesicular transport suggests that this could be regulated by changes in the driving force of transport (i.e. the H^+ electrochemical gradient), the concentration of cytosolic neurotransmitter, or regulation of the transporter itself. In fact, increasing the cytosolic concentration of transmitter leads to increases in the vesicular storage of transmitter. This is seen most clearly as the basis for the therapeutic efficacy of leva-dopa, a widely proscribed treatment in Parkinson's disease. Administration of leva-dopa, the synthetic precursor of dopamine, alleviates symptoms associated with a loss of dopaminergic terminals in PD. The efficacy of this treatment is due to an elevation of cytosolic levels of dopamine (synthesized intracellularly from leva-dopa), and a subsequent increase in the vesicular storage and quantal size of DA release (Emmanuel Pothos, 1996). The ability to alter the vesicular content of secretory vesicles however, relies most directly on the VNTs. The copy number, location and activity of VNTs all have the potential to regulate quantal size. In the following section, the growing evidence for the regulation of VNTs will be presented.

1.2.3 Regulation of VNTs

Because, quanta were largely considered invariant, the relevance of VNT regulation to neurotransmission had to first be demonstrated. The most direct and convincing evidence to show that alterations of VNT function led to changes in quantal size and neurotransmission were experimental manipulations of VNT copy number. Overexpression of VAChT or $VMAT_2$ was demonstrated to increase quantal size (Pothos et al., 2000; Song et al., 1997). Interestingly, expression of $VMAT_2$ in cells or neurons that do not normally store or release catecholamines was able to induce quantal release of dopamine (Li et al., 2005; Pothos et al., 2000). On the

other hand, knock-down of $VMAT_2$ led to reductions in monoamine transmission. Animals in which $VMAT_2$ expression was greatly reduced but not eliminated were viable but showed behavioral deficits including motor deficits and a depressive–like phenotype (Fukui et al., 2007; Mooslehner et al., 2001). Another study examining $VMAT_2$ deficient animals demonstrated age-dependent progressive loss of substantia nigra dopaminergic neurons, characteristic of Parkinson's disease (PD), and an increased accumulation of the PD related protein alpha-synuclein (Colebrooke et al., 2006). Knockdown of VAChT in mice led to impairments of cholinergic transmission and deficits in learning and memory tasks (de Castro et al., 2008; Prado et al., 2006). These studies demonstrated that behaviorally relevant changes in neurotransmission were induced by alterations of vesicular transport levels, thus identifying VATs as an important potential site of regulation.

Not surprisingly, recent studies have begun to implicate alterations of VATs in disease pathogenesis. In Huntington's disease (HD) a downregulation of VAChT protein independent of neuronal death is seen in post-mortem HD human tissue as well as in a mouse model of HD (Smith et al., 2006). Consistently, changes in neurotransmission have been implicated as some of the earliest changes in HD pathogenesis (Smith et al., 2005). Alpha-synuclein, a protein implicated in familial PD and seen to accumulate in neurons during sporadic PD, can interact directly with $VMAT_2$ (Guo et al., 2008). In addition, *in-vitro* overexpression of the wildtype or mutant alpha-synuclein protein leads to a decrease in $VMAT_2$ protein levels and an increase in cytosolic dopamine levels (Mosharov et al., 2006). Strikingly, an increase in cytoplasmic dopamine is thought to contribute greatly to neurodegeneration of neurons in PD (Hastings and Zigmond, 1997).

Regulation of VNTs have also been seen after acute application of drugs of abuse (For review see Fleckenstein and Hanson, 2003). In particular, alteration of $VMAT_2$ function is known to play a major role in the psychotropic effects of drugs of abuse, including amphetamines. Amphetamines induce the rapid depletion of monoamines from synaptic vesicles by direct interaction with VMAT (Partilla et al., 2006) as well as their actions as a weak base (Sulzer et al., 1992). Moreover, amphetamines have been recently linked to changes in VMAT localization. Purified cytoplasmic vesicle preparations from synaptosomes of animals exposed to a single high dose of methamphetamine showed decreased binding of $VMAT_2$ markers as well as decreased monoamine uptake (Hogan et al., 2000). This was consistent with studies that showed that methamphetamine decreased $VMAT_2$ protein levels in a cytoplasmic vesicle preparation without a change in total homogenate. This indicated a redistribution of $VMAT_2$ protein away from vesicles (Riddle et al., 2002). On the other hand, cocaine, an inhibitor of the plasma membrane DAT, led to a shift in $VMAT_2$ localization to cytoplasmic vesicle-enriched fractions and a concomitant increase in vesicular dopaminergic uptake. While the mechanisms of this drug-induced regulation of $VMAT_2$ are not clear, the cocaine effect is blocked by antagonists to the D_2 dopamine receptor suggesting that it is regulated through a metabotropic signaling cascade (Brown et al., 2001; Riddle et al., 2002).

The above studies suggest that regulation of VNTs may be a relevant mechanism for changes in neurotransmission related to disease and drugs of abuse. Recent studies have begun to identify other potential mechanisms of VNT regulation that may play a role in more physiologic regulation of neurotransmission. In a series of studies done in both cell lines and primary neurons, Ahnert-Hilger and colleagues have shown that $VMAT_1$ and $VMAT_2$ as well as VGluT can be regulated by heterotrimeric G proteins that associate with the secretory vesicle.

Using transport assays from isolated vesicle preparations of neuroendocrine cells and primary neurons, uptake of radiolabelled monoamines was inhibited by $Go_2\alpha$. The electrochemical gradient of vesicles is not altered, suggesting direct modulation of $VMAT_2$ function. Interestingly, the intralumenal loop of the transporter and packaged transmitter are implicated in the G protein inhibition as a means to sense and regulate vesicular content. Although the role of G protein mediated VAT regulation is not yet understood, it is the first identification of regulatory machinery that directly alters VAT function (Ahnert-Hilger et al., 1998; Brunk et al., 2006; Holtje et al., 2000; Holtje et al., 2003).

Direct demonstration of physiologic regulation of VNTs comes from recent studies that examined the periodicity of VGluT expression on synaptic vesicles (Darna et al., 2008; Yelamanchili et al., 2006). Studies found that while the total protein level of VGluT in mouse brain was constant throughout the day, the amount present on synaptic vesicles was strongly regulated by circadian rhythm. Using a pronase assay, the authors demonstrated that levels of plasma membrane localized VGluT, but not other synaptic vesicle proteins such as synaptotagmin, fluctuated depending of the time of day. In a complementary fashion, isolated secretory granules showed shifting levels of the vesicular transport of glutamate, suggesting a diurnal translocation of VGluT from the plasma membrane to synaptic vesicles. This regulation required the Per2 gene, implicated in light adaptations of the biological clock as animals lacking the gene did not show this VGluT regulation. These studies show that the VNTs are a target of physiologic regulation. Moreover, this regulation is capable of defining quantal size and neuronal signaling properties and is relevant to disease pathogenesis, drug effects and plasticity of neurotransmission.

1.3 VESICULAR NEUROTRANSMITTER TRANSPORTER TRAFFICKING

Regulation of protein trafficking has recently been identified as a primary mechanism of acute regulation of transmission. For example the membrane trafficking of AMPA receptors are believed to underlie early changes in synaptic plasticity (Malenka, 2003). Recent studies suggest that the regulation of VNT trafficking is also involved in defining characteristics of neurotransmitter release, neuronal function and behavior. During both the biogenesis and the activity-dependent recycling of secretory vesicles, VNTs undergo trafficking that has the potential to determine both the type of secretory vesicle into which neurotransmitter is packaged as well as the amount of neurotransmitter packaged into vesicles. Thus, VNT trafficking is amenable to regulation as a means of defining characteristics of synaptic transmission. Understanding the signals, mechanisms and regulation of VNT trafficking are therefore essential to understanding neurotransmission.

1.3.1 Secretory Vesicle Types

Multiple types of secretory vesicles with distinctive release properties underlie regulated release from neurons. The targeting of vesicular transporters to distinct types of vesicles defines their neurotransmitter content and thus characteristics of neurotransmitter release. The primary types of secretory granules that mediate neurotransmission are small clear synaptic vesicles (SVs) and large dense core vesicles (LDCVs). In addition small dense core vesicles (SDCVs) are often categorized as a unique vesicle class although they are poorly characterized. Many characteristics including size, origin and content of these classes of vesicles are unique, as are their release properties and role in neuronal signaling (Edwards, 1998; Martin, 2003). SVs,

morphologically characterized as small (~ 40-50 nm) are typically found in clusters at specialized regions of membrane termed active zones and mediate the fast synaptic transmission of classical transmitters. The properties of activity-dependent SV release have been characterized extensively at the frog neuromuscular junction with the release of Ach and in central hippocampal synapses with the release of glutamate. Classically, these vesicles show high Ca^{+2} sensitivity, synchronous release and short release latency (< 1 ms). These vesicles recycle locally at release sites, rapidly refill and can undergo multiple rounds of release. LDCVs, on the other hand, are characterized by their larger size (> 80 nm) and electron dense neuropeptide content. In addition to neuropeptides, LDCVs often contain monoaminergic transmitter. The release properties of LDCVs, classically studied using chromaffin granules, show lower sensitivity for Ca^{+2}, release more slowly (> 10 ms) and show asynchrony of release. Moreover, LDCVs are not thought to recycle locally, but rather form at the TGN where they are filled with neuropeptide content.

The subcellular distribution of VATs on secretory vesicles has been studied in some systems, however characterization is far from complete. Although cholinergic terminals contain both SVs and LDCVs, VAChT preferentially localizes to SVs (Gilmor et al., 1996; Weihe et al., 1996). This preferential localization to SVs makes it an ideal candidate protein to study the signals and machinery that regulate SV-specific trafficking. On the other hand, the VMAT transporter has been localized to multiple vesicles types including SVs, LDCVs, and SDCVs (Nirenberg et al., 1996). The localization of VMAT to multiple vesicle populations suggests that its trafficking may be regulated between vesicle types.

1.3.2 Vesicle Specific Targeting of VATs

The regulated targeting of VATs to specific secretory vesicles occurs primarily during secretory vesicle biogenesis. In neurons, newly synthesized proteins are trafficked through a multiple step process including sorting at the TGN into constitutive vs. regulated pathways, axonal versus dendritic targeting, and secretory vesicle maturation. Evidence regarding the sequence and mechanisms of these events are not clear and it is likely that these steps are unique for individual proteins. However, some properties seem to be well generalized. The targeting of newly synthesized proteins to either SVs or LDCVs seems to be regulated primarily at the level of the trans Golgi network (TGN). SV bound proteins are thought to transit from the TGN to the plasma membrane by constitutive exocytosis. At the plasma membrane SV proteins undergo internalization and sorting to form mature SVs (Figure 3A). Proteins destined for LDCVs, on the other hand, are sorted to the regulated pathway of secretion. This sorting is thought to occur through two mechanisms: (1) sorting by entry, proteins are selectively sorted into the regulated secretory pathway and enter immature secretory granules (ISGs); (2) sorting by retention, LDCV proteins are retained during maturation of ISGs while other proteins are removed. The maturation of ISG to LDCVs occurs via the budding-off of proteins destined for other subcellular organelles (Tooze and Stinchcombe, 1992).

During activity dependent recycling of secretory vesicles, endosomal compartments may also play a role in sorting of LDCV and SV proteins. Components of both vesicle-types are seen intermixed in early endosomes after stimulation (Partoens et al., 1998). Sorting of proteins back to the TGN may enhance their targeting to LDCVs while proteins destined for SVs are likely recycled locally.

Much of what is known about the molecular signals that mediate the trafficking of VATs has come from biochemical analysis of nascent proteins in cell lines, namely the neuroendocrine PC12 cell line. This cell line, expresses low levels of both VAChT and $VMAT_1$. In PC12 cells VAChT localizes strongly to synaptic vesicle like vesicles (SVLVs), whereas exogenous expression of $VMAT_2$ or endogenous $VMAT_1$ preferentially localize to LDCVs. The preferential localization of these two similar proteins to unique secretory vesicle types has made this an advantageous system for studying the signals and machinery that regulate vesicle-specific traffic. Studies investigating the trafficking of these proteins have used chimera between $VMAT_2$ and VAChT in order to identify regions of the protein important for vesicle specific targeting. Chimera in which the cytoplasmic tail of VAChT and VMAT were switched demonstrated the importance of the C-terminus to its localization. Mutation analysis within these regions identified classic dileucine motifs that were essential to internalization of both VAChT and VMAT (Tan et al., 1998). Upstream glutamate residues of the $VMAT_2$ dileucine motif (K**EE**KMAIL) were shown to be involved in the specific localization of $VMAT_2$ to LDCVs. Mutation of these residues to alanines reduced VMAT2 targeting to LDCVs without altering its endocytosis. Interestingly, phosphorylation of a serine residue upstream of the VAChT dileucine motif (R**S**ERDVLL), which mimics the negative charges of the $VMAT_2$ upstream residues, has been shown to promote trafficking of VAChT to LDCVs (Krantz et al., 2000). Furthermore, an acidic patch in the C terminus of $VMAT_2$ has been identified as a retention sequence for localization to LDCVs. The deletion of these residues or the phosphorylation of two serine residues within this patch by casein kinase 2 promotes the removal of $VMAT_2$ from immature granules during maturation and thus reduces its expression on LDCVs (Waites et al., 2001). These mechanisms suggest that changes in phosphorylation states of VATs

may play an important role in their regulated targeting. The physiological regulation and relevance of this potential regulation remains to be investigated in neurons. Moreover, the cytosolic machinery that may regulate these trafficking events has not been identified.

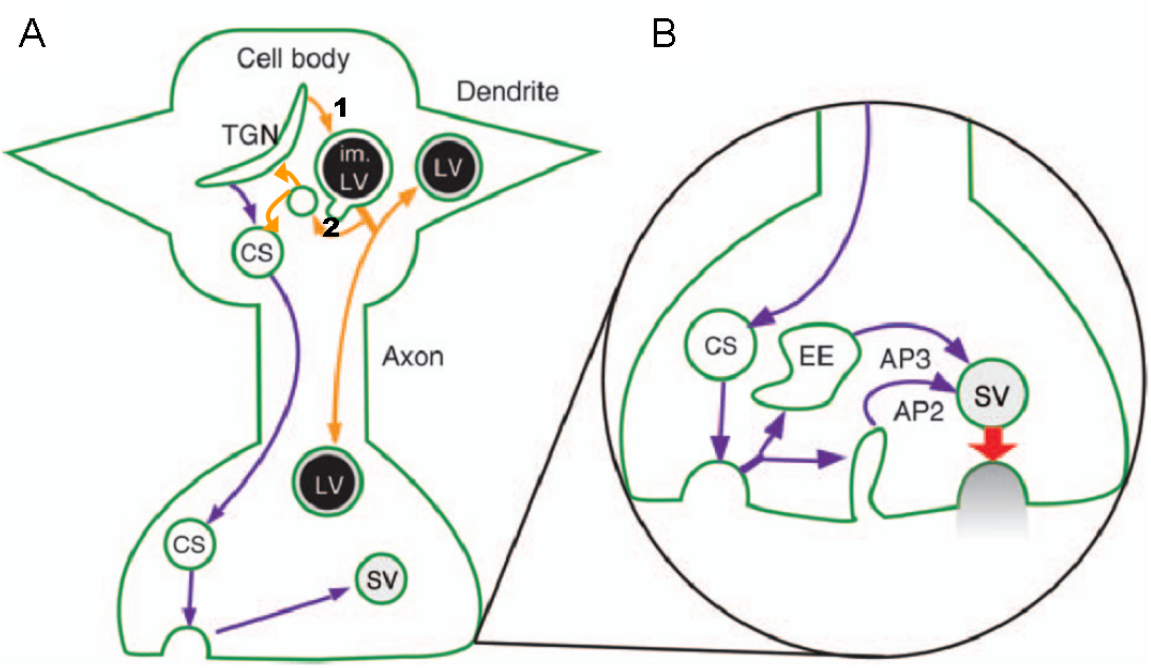

Figure 3. Secretory Vesicle Biogenesis

A. Vesicle Specific Biogenesis. Large dense core vesicles (LV) are sorted by selection at the TGN (1) or retention (2) during immature granule (im. LV) maturation. Synaptic vesicles (SV) are formed after internalization from components that have undergone constitutive secretion (CS). B. Synaptic Vesicle Formation. At the terminal SVs are formed by internalization through an endosomal intermediate (EE) in an AP-3 dependent manner and/or via direct internalization though an AP-2 dependent mechanism. (Modified from Fei et al., 2008).

1.3.3 Synaptic Vesicle Biogenesis and Recycling

Data examining axonal transport of tubulovesicular structures carrying SV proteins suggest that SV destined proteins are transported individually or in subsets through constitutive exocytosis or in specialized synaptic vesicle precursors (Okada et al., 1995). Regardless, the formation of the mature SV, containing the required complement of SV proteins and characteristic morphology, occurs through internalization at the nerve terminal. Internalization may be mediated by direct budding of the SV from the plasma membrane in an AP-2, clathrin dependent manner or budding from an endosomal intermediate requiring AP-3 (Figure 3B; for review see Hannah et al., 1999). The central role of clathrin-mediated internalization in the formation of SVs suggests that SV biogenesis may share common mechanisms of protein targeting with those of activity-dependent SV recycling.

Mechanisms of SV recycling were first characterized through the publication of several seminal papers in the 70's (For review see Heuser, 1989). These experiments used electron microscopy to visualize neuromuscular junction synapses that had been stimulated in the presence of a fluid phase marker to label recycling vesicles. After stimulation labeled vesicles were detected, indicating the vesicle recycling. However, the mechanisms of recycling proposed by different group were quite distinct (Figure 4). In one case, Ceccarelli and colleagues reported the appearance of labeled vesicles with no change in the number of vesicles or the size of the membrane even after hours of stimulation. Moreover, clathrin intermediates were not identified, suggesting a clathrin-independent form of recycling (Ceccarelli et al., 1973). Heuser and Reese, however reported that after just minutes of stimulation, a rapid depletion in the number of synaptic vesicles was seen, concomitant with an increase in the size of the plasma membrane. Moreover the appearance of many labeled cisternae and clathrin coated vesicles were evident.

Heuser, therefore, concluded that endocytosis was mediated by clathrin coated vesicles that fused with cisternae. Synaptic vesicle were formed by budding off of these cisternae (Heuser and Reese, 1973). In the first proposal, later termed 'kiss and run', the vesicle appears to recycle locally and rapidly as if vesicular components remain segregated from the plasma membrane. Exocytosis is achieved by the opening of a fusion pore that can be closed resulting in the immediate reformation of the vesicle. The vesicle either remains docked at the active zone or cycles into a larger pool of vesicles available for reuse. The second proposal however suggested that exocytosis occurs by full fusion. This is accompanied by intermixing of vesicular components with the target membrane (Li and Murthy, 2001). Recycling subsequently requires the sorting of appropriate membrane and protein components through interaction with adaptors, including AP-2, before internalization in clathrin-coated vesicles. Internalized vesicles may uncoat to directly form synaptic vesicles or may form from an endocytic intermediate from which they bud by an AP-3 dependent mechanism.

The coexistence of these two pathways was revealed in retinal neurons of the Drosophila shibire mutant. This mutant, a TS disruption of dynamin, revealed two modes of vesicle recycling. The first, a direct pinching off of SVs, or 'kiss and run', was seen at the active zone and mediated fast recycling. Concurrently, a slower recycling pathway was observed away from active zones and involving complex branching structures (Koenig and Ikeda, 1996). The prevalence of these different recycling pathways seems to depend in part on the duration and/or intensity of neuronal stimulation, with fast, clathrin-independent recycling at low frequency stimulation and clathrin-dependent, or bulk endocytosis, at high frequency stimulation (de Lange et al., 2003).

The regulation between and physiologic relevance of multiple mechanisms of vesicle recycling is not clear. However several hypotheses have been suggested. The activity dependence of the different pathways may suggest that bulk endocytosis occurs only under strong stimulus conditions that may saturate fast, clathrin independent modes of recycling. An alternative suggestion is that different recycling pathways may form distinct vesicle pools and/or distinct synaptic vesicles. In this case, vesicles undergoing rapid endocytosis recycle locally to the ready-releasable pool of vesicles, whereas slower endosomal mediated pathways recycle to a reserve pool of vesicles (Richards et al., 2000). A third consideration is the apparent preference of certain SV proteins to recycle through specific pathways. The mocha mouse, which displays a mutation in the AP-3 adaptor protein, involved in the endosomal formation of SVs, shows selective mislocalization of certain SV proteins (including the VIAAT and the Zn transporter) with no apparent defects in other SV proteins. These results suggest that trafficking of selective SV proteins is mediated in an AP-3 dependent manner (Kantheti et al., 1998; Nakatsu et al., 2004). Although many questions remain as to their precise physiologic relevance, it is clear that the presence of multiple trafficking pathways allows for regulation of protein trafficking during SV recycling.

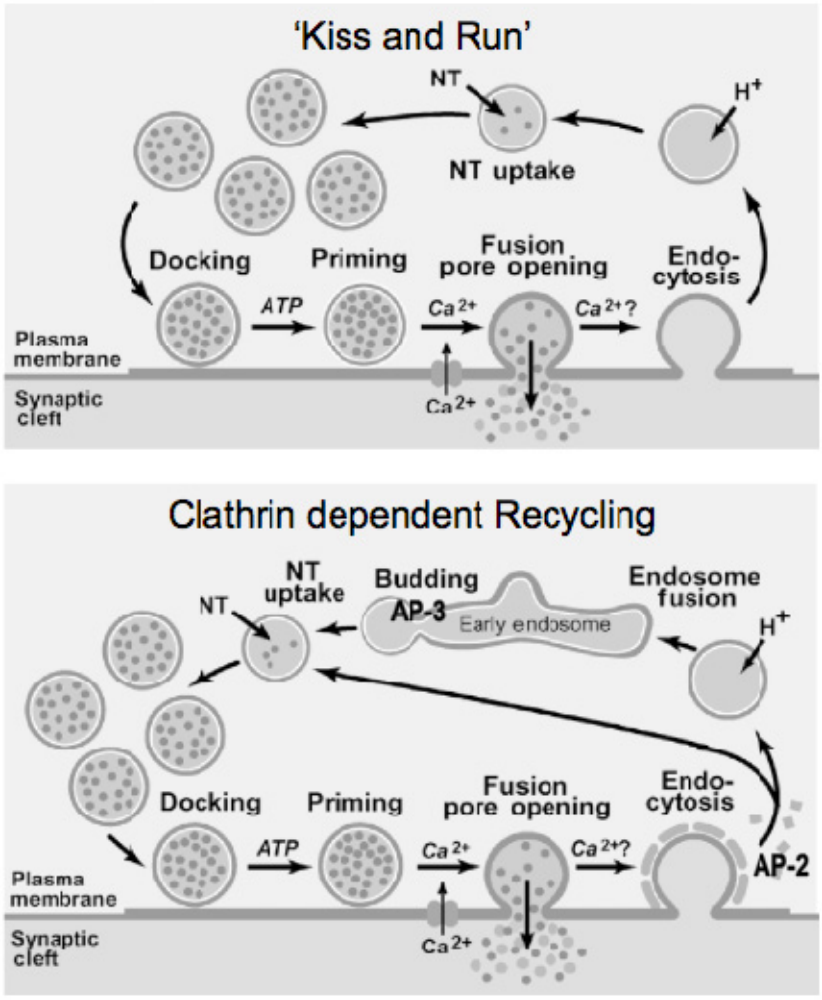

Figure 4. Synaptic vesicle recycling pathways

Multiple pathways are proposed: (1) kiss-and-run- a pathway in which vesicles endocytose by closure of the fusion pore and are refilled with neurotransmitters either while remaining docked to the active zone or via a local recycling pathway that is clathrin independent but results in mixing vesicles with the reserve pool after endocytosis; (2) clathrin mediated endocytosis- a pathway whereby vesicles undergo clathrin-mediated endocytosis and recycle either by direct uncoating or via an endosomal intermediate. (Modified from Sudhof, 2004).

1.3.4 Synaptic Vesicle Targeting of VATs

The selective localization of VAChT to SVs has made it an ideal protein for identifying synaptic vesicle specific targeting sequences. The central role of internalization in the biogenesis and recycling of synaptic vesicles suggests that signals that mediate the internalization of SV proteins should be important in their SV targeting. Consistently, the dileucine motif in VAChT is required for its SV targeting (Tan et al., 1998). Furthermore, VAChT has been shown to interact with clathrin adaptor protein AP-2, involved in clathrin mediated endocytosis. Moreover disruption of endocytic machinery such as dynamin and clathrin leads to accumulation of VAChT on the plasma membrane (Barbosa et al., 2002; Ferreira et al., 2005) and thus a reduction in its SV targeting. In addition to interaction with AP-2, an interaction with the adaptor protein AP-1, normally associated with trafficking at the TGN, has been indicated, although the functional significance of this interaction is not yet known. Furthermore, a non-traditional tyrosine motif has also been implicated in the internalization of VAChT, although it is not required in neuroendocrine cells. Interestingly, an interaction between VAChT and AP-3 has not been detected, thus alternative machinery may be involved in the endosomal trafficking of VAChT to synaptic vesicles (Ferreira et al., 2005). The requirement of endocytosis for the SV targeting of VAChT is clear, however the presence of a sufficient SV targeting motif is unknown. Moreover, the proteins that regulate the SV specific targeting of VAChT are unknown.

1.4 CURRENT ASSAYS OF VNT FUNCTION

Recent evidence suggests that vesicular storage may be regulated under physiologic conditions, during exposure to drugs and during the pathogenesis of disease. Because alterations of vesicular storage lead to changes in synaptic transmission, understanding this regulation is essential. However, the inability to directly monitor vesicular transport in living neurons has hindered understanding of VNT function and regulation during neurotransmission.

Biophysical measurements of VNT mediated transport have relied on *in-vitro* transport assays. These studies have been invaluable for understanding mechanisms of transport including molecular and chemical properties. However the necessity of isolating vesicular fractions in these assays unavoidably isolates the transport process from the cellular environment as well as separates vesicle transport from vesicular release. Thus, while in-vitro transport assays are a direct measurement of VNT function, they do not allow the acute study of physiologic regulation of transport or the relationship of alterations in transport with neurotransmission.

Electrophysiological assays, on the other hand, are able to dynamically assess changes in quantal size but are not able to directly attribute these changes to alterations in vesicular transport. Electrophysiology measures postsynaptic cellular responses to stimulation. Therefore changes in quantal size can be indicative of changes in presynaptic or postsynaptic mechanisms. Furthermore as the readout is the summation of all inputs into the selected cell, this technique provides poor spatial information and localized changes (i.e. at a single or subset of terminals) may not be detected.

Recently, imaging techniques have allowed the exploration of subcellular dynamics of vesicular proteins and membranes. The use of fluorescently tagged proteins and lipophilic dyes has provided a means to study the trafficking of SV proteins and vesicles with good spatial and

temporal resolution. However, while providing valuable information on the vesicle cycle they are unable to provide information on vesicular content or the function of vesicular transporters.

Perhaps the most informative studies thus far have relied on perturbation of transporter function through drugs or genetic techniques followed by one of the readouts assays described above. While these approaches stress the essential role of vesicular transport in neurotransmission and are able to identify the potential for VNT regulation to modulate neuronal function, they preclude the understanding of normal VNT function and regulation during neurotransmission.

A live assay of vesicular transport in neurons would aid in our understanding of the role of vesicular packaging in neurotransmission. By taking advantage of optical techniques to visualize neurotransmitter, vesicular transport can be studied with good spatial resolution and minimal perturbation of the system. An assay that monitors neurotransmitter would allow for measurements of both vesicular transport as well as release. This would provide insight into both the normal function of VNTs in the context of neurotransmission, allowing for the first measurements of the contribution of activity dependent vesicular transport to release. Moreover, this would provide a means of assaying VNT regulation and the role of this regulation in shaping properties of neurotransmission.

1.5 THESIS GOALS

Until recently, the regulation of VNTs as a means to modulating synaptic transmission had been underappreciated. Thus, many questions remain about the regulation of VNTs as well the consequences of VNT regulation for neurotransmission. To understand complex mechanisms of VNT regulation an understanding of basic properties, such as VNT trafficking and the contribution of vesicular transport to release are necessary. Thus, the goals of this thesis were (1) to better understand the signals and machinery that mediate the SV-specific trafficking of vesicular transporters and (2) to establish a live-cell optical assay to measure vesicular transport and its contributions to release in neurons.

The results described in Chapter 2 rely on the synaptic vesicle specific targeting of VAChT in a neuroendocrine cell line to identify a sufficient synaptic vesicle targeting motif. The identified motif contains a classical dileucine motif that shows duality of function as an internalization and synaptic vesicle targeting sequence. The specificity of this motif as a SVTM is discussed. This work has been previously published (Colgan et al., 2007).

In Chapter 3 Sorting nexin 5 (SNX5) is identified as a novel regulatory protein that directs the SV trafficking of VAChT. SNX5 is characterized and the functional interaction of SNX5 and VAChT is tested. Disruption of SNX5 leads to the mistargeting of protein from SVs to LDCVs. This chapter has been written in the format of a 'Brief Communication' in preparation for publication.

In Chapter 4 a novel assay is established that allows for the first measurements of vesicular transport in live neurons. Concomitant measurements of vesicular packaging and release allow for the contributions of vesicular transport to release to be assessed. This work is in preparation for publication.

Chapter 5 presents a summary of the work presented and a broad discussion of its implications for the field of VNTs and the larger field of neurotransmission. The incorporation of the findings within a broader view of the literature and future directions for study are addressed. Throughout, I focus on the active role of VNTs in shaping the properties of neurotransmission.

2.0 DILEUCINE MOTIF IS SUFFICIENT FOR INTERNALIZATION AND SYNAPTIC VESICLE TARGETING OF VESICULAR ACETYCHOLINE TRANSPORTER

2.1 ABSTRACT

Efficient cholinergic transmission requires accurate targeting of vesicular acetylcholine transporter (VAChT) to synaptic vesicles (SVs). However, the signals that regulate this vesicular targeting are not well characterized. Although previous studies suggest that the C-terminus of the transporter is required for its SV targeting, it is not clear whether this region is sufficient for this process. Furthermore a synaptic vesicle targeting motif (SVTM) within this sequence remains to be identified. Here we use a chimeric protein, TacA, between an unrelated plasma membrane protein, Tac, and the C-terminus of VAChT to demonstrate the sufficiency of the C-terminus for targeting to synaptic vesicle-like vesicles (SVLVs) in PC12 cells. TacA shows colocalization and cosedimentation with the SV marker synaptophysin. Deletion mutation analysis of TacA demonstrates that a short, dileucine-motif containing sequence is required and sufficient to direct this targeting. Di-alanine mutation analysis within this sequence suggests indistinguishable signals for both internalization and synaptic vesicle sorting. Using additional chimeras as controls, we confirm the specificity of this region for SVLVs targeting. Therefore, we suggest that the dileucine containing motif is sufficient as a dual signal for both internalization and SV targeting during VAChT trafficking.

2.2 INTRODUCTION

Synaptic transmission requires the efficient vesicular packaging of neurotransmitters, a process mediated by a group of vesicular neurotransmitter transport proteins (VNT) (Liu and Edwards, 1997). Thus, targeting of these functional transport proteins to synaptic vesicles (SVs) is highly regulated in order to maintain synaptic efficacy. Despite this, the molecular mechanisms underlying the SV targeting of VNTs remain elusive. Evidence suggests that clathrin mediated internalization plays an important role in the targeting of both newly synthesized and recycling VNTs to synaptic vesicles. From the cell surface, SV bound proteins require internalization and sorting that is thought to occur through at least two distinct pathways. SV proteins may undergo selective internalization to directly form SVs, or proteins may be indiscriminately internalized to an endosomal intermediate from which SV proteins can be selectively sorted (Hannah et al., 1999; Sudhof, 2004). The regulation of these distinct sorting processes, although not well understood, is believed to be mediated through specific cytoplasmic sorting motifs. These motifs execute their role in membrane trafficking through interaction with corresponding cytosolic machinery that direct the protein accordingly. Sorting- motifs for either internalization or SV targeting (SVTM) have been identified in several SV proteins (Blagoveshchenskaya et al., 1999; Grote et al., 1995; Han et al., 2004; Pennuto et al., 2003; Prado and Prado, 2002). For example, synaptophysin has been shown to contain a C-terminal repeating tyrosine based internalization motif that recruits dynamin to the membrane and directs clathrin-independent internalization(Daly et al., 2000; Daly and Ziff, 2002; Pennuto et al., 2003). Another SV protein, synaptotagmin1, has a cytoplasmic dileucine-motif which is required for AP-3 mediated SV targeting (Blagoveshchenskaya et al., 1999). In contrast, another study suggested a role for synaptotagmin's lumenal N-terminus in SV targeting (Han et al., 2004). While specific motifs

have been indicated to play a role in SV targeting, the sufficiency of these motifs as well as the generality of these signals is not known.

Vesicular Acetylcholine Transporter (VAChT) is a member of the VNT family that is responsible for packaging acetylcholine into vesicles for regulated release (Alfonso et al., 1993; Rand, 1989; Roghani et al., 1994). Molecular manipulations to alter the vesicular level of VAChT on SVs, namely overexpression or knockdown, lead to corresponding alterations in cholinergic transmission (Kitamoto et al., 2000; Song et al., 1997). Although a specific SVTM has yet to be identified, the C-terminus of VAChT has been found to be required for its vesicular localization (Tan et al., 1998; Varoqui and Erickson, 1998). Interestingly, this region has been identified to be phosphorylated by PKC, which was suggested to play a role in modulating its internalization or trafficking (Cho et al., 2000; Krantz et al., 2000). Furthermore, a classical dileucine motif within this region has been identified to be required for the internalization of the transporter (Tan et al., 1998). Interaction of VAChT through its C-terminus with clathrin associated machinery including AP-1, and AP-2 further suggest a critical role of the cytoplasmic sequence in the regulated clathrin mediated internalization and membrane trafficking of VAChT (Barbosa et al., 2002; Kim and Hersh, 2004). Consistently, interference with internalization machinery, including clathrin and dynamin, or mutation of the internalization motif, disrupt the targeting of VAChT to SVs leading to an accumulation of the protein on the cell surface (Barbosa et al., 2002; Ferreira et al., 2005; Tan et al., 1998). Together, these findings suggest a correlation between the C–terminal directed, regulated internalization of the transporter and its SV targeting. However, whether the C-terminus of VAChT is sufficient for SV targeting remains unknown. Furthermore, it is unclear how the internalization of VAChT is involved in its

vesicular localization and most importantly, whether this SV targeting is determined by a SVTM within this region.

Here we report, by using a chimeric protein between an unrelated plasma membrane localized protein, Tac, and the VAChT C-terminus, the sufficiency of this sequence in synaptic vesicle like vesicle (SVLV) targeting in PC12 cells. We further characterize the dileucine containing motif of VAChT as a SVTM and examine its role as a dual signal for both internalization and SVLV targeting.

2.3 RESULTS

2.3.1 The C-terminus of VAChT is sufficient for SVLV targeting

The C-terminus of VAChT has been shown to be required for its preferential localization to SVLVs in PC12 cells (Tan et al., 1998). Chimeric analysis between VAChT and a closely related, but preferentially large dense core vesicle (LDCV) localized VNT, vesicular monoamine transporter 2 ($VMAT_2$), has suggested that the VAChT C-terminus is able to direct $VMAT_2$ to SVLVs (Tan et al., 1998). However, these results do not distinguish whether the VAChT C-terminus is sufficient for SVLV targeting. To test this possibility, we took advantage of Tac protein, the α-subunit of the interleukin 2 receptor (IL-2R), which has a single membrane spanning domain whose internalization requires the presence of additional IL-2R subunits (Letourneur and Klausner, 1991; Marks et al., 1995; Tan et al., 1998). Not endogenously expressed in PC12 cells, Tac protein localizes to the plasma membrane when exogenously introduced (Figure 5). Through fusing the C-terminus of VAChT to Tac, we created a chimeric

protein, TacA (Figure 5A), which would allow us to independently examine the trafficking properties of the VAChT C-terminus in the context of an unrelated protein.

In order to determine whether the C-terminus of VAChT is sufficient to target Tac to SVLVs, stable PC12 transformants expressing Tac protein or the chimera TacA were examined. Through immunofluorescence staining, Tac protein showed diffuse plasma membrane localization with very little cytoplasmic distribution (Figure 5B). In contrast, the chimeric TacA showed localization to internal punctate structures enriched in both the perinuclear and tip regions of differentiated PC12 cells (Figure 5B). These TacA puncta were found to colocalize with endogenous synaptophysin, a SV marker, but not secretogranin II, a LDCV marker (Figure 5B). Furthermore, biochemical analysis of Tac and TacA through gradient sedimentation (Bauerfeind et al., 1993) showed that TacA localizes primarily to the light fractions of density sucrose gradients and co-sediments preferentially with synaptophysin (72.17 +/- 1.557%), but not secretogranin II (Figure 5D). In contrast, cells expressing wild type plasma membrane localized Tac protein sedimented to heavier fractions (8-10) than SVLV containing fractions (9-12) (Figure 5C). Using velocity gradient fractionation, designed to isolate SVLVs by excluding other larger organelles such as endosomes (Clift-O'Grady et al., 1998; Clift-O'Grady et al., 1990; Liu et al., 1994), TacA is found in SVLV containing fractions, strongly indicating its SVLV targeting (Figure 5F). As expected, Tac is excluded from these SVLV containing fractions and localizes to the bottom of the gradient (Figure 5E). These findings were also confirmed with transiently transfected PC12 cells to avoid potential problems associated with stable lines (data not shown). Thus, three independent assays indicate the localization of TacA to SVLVs, confirming the sufficiency of the VAChT C-terminus to target an unrelated protein to SVLVs and suggesting that this region contains a SVTM.

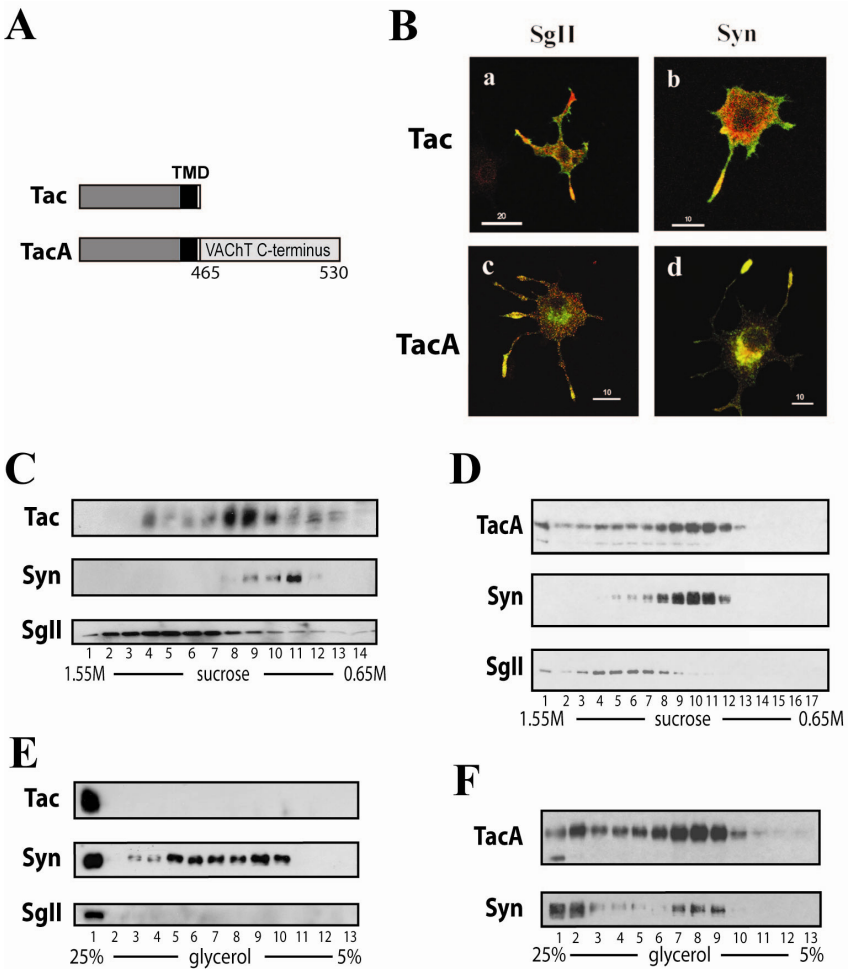

Figure 5. VAChT C-terminus is sufficient for SVLV targeting in PC12 cells.

A) Schematic diagram of TacA construct. VAChT C-terminus was fused to the C-terminal end of Tac protein. B) Targeting of TacA to SVLV using imaging analysis. PC12 cells stably expressing Tac and TacA were visualized through immunofluorescence (green). SVLVs and LDCVs were identified with antibodies against endogenous synaptophysin (Syn) or secretogranin II (SgII), respectively (red). C) Tac does not target to SVLVs in PC12 cells. Overexpressed Tac proteins in transiently transfected PC12 cells were fractionated through a sucrose density gradient (0.65 M-1.55 M). D) Targeting of TacA to SVLVs using density sedimentation. PNS from TacA stable PC12 cells was fractionated through a sucrose density gradient. E) Exclusion of Tac from SVLV isolating velocity gradient. PNS from transiently transfected cells was fractioned through a glycerol velocity gradient (5%-25%). F) Targeting of TacA to SVLVs using velocity sedimentation. Glycerol velocity gradient was used for fractionation of TacA stable PC12 cells.

2.3.2 Dileucine containing motif is required for SVLV targeting

The cytosolic tail of VAChT consists of 60 amino acids which contain several previously defined motifs including a classic dileucine motif [E(XXX)LL], a classical tyrosine motif (YXXØ), and a nonclassical tyrosine motif. In order to investigate which of these motifs determines the SVLV targeting of VAChT, a series of TacA chimera with deletions within the C-terminus of VAChT were constructed (Figure 6A). These chimeras were then examined for their subcellular localization in PC12 cells. Targeting to SVLVs was determined through two criteria: steady-state colocalization with synaptophysin through immunofluorescent staining, and comigration with SVLV containing fractions in velocity subcellular fractionation analysis. As shown in figure 6B, constructs that met both of these criteria are indicated with a symbol [+] for their SVLV targeting. In contrast, constructs unable to target to SVLVs are indicated with a symbol [--] for SVLV targeting. Analysis of deletion mutants clearly indicated the requirement of a small dileucine motif containing region (Figure 6A, indicated in grey) for SVLV targeting. The deletion of this region disrupted SVLV targeting and led to the accumulation of the mutant on the cell surface, presumably due to a loss of dileucine motif mediated internalization. This is demonstrated through representative immunofluorescent images in Figure 6C. Deletion mutant analysis suggested that the dileucine containing motif is the only region required for SVLV targeting. Other regions, previously reported to play a role in VAChT trafficking, including tyrosine motifs (Kim and Hersh, 2004), or the phosphorylation at serine 480 (Cho et al., 2000; Krantz et al., 2000) were not found to be essential for SVLV targeting in our system. Thus the 10 a.a. dileucine motif containing sequence of the VAChT C-terminus is essential for the SVLV targeting of VAChT.

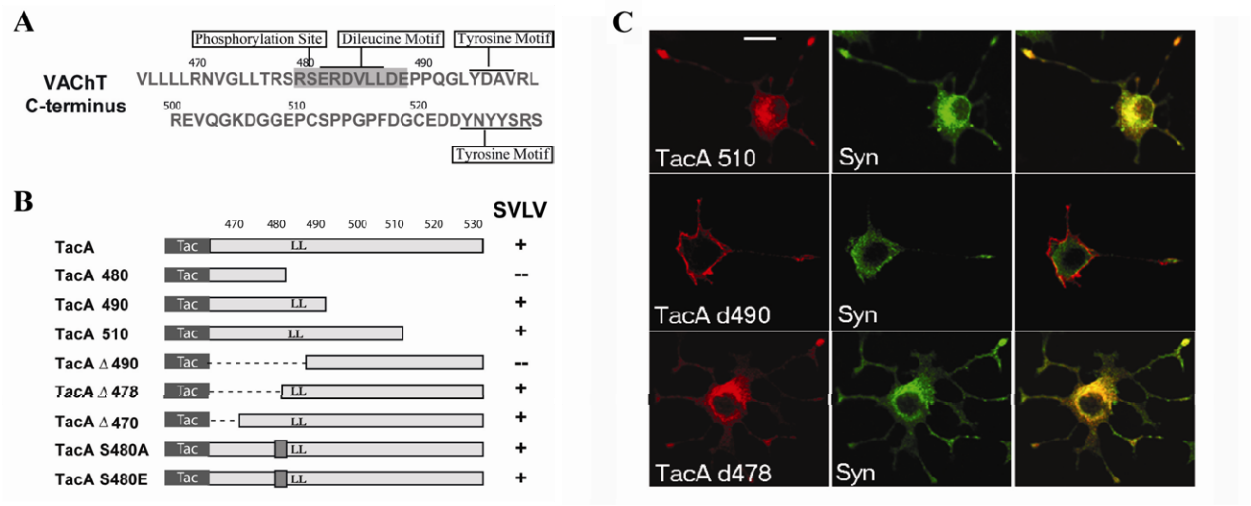

Figure 6. Dileucine containing motif is necessary for SVLV targeting.

A) Amino acid sequence of VAChT C-terminus (residues 465 to 530). Dileucine containing motif is shaded in grey. B) Targeting of deletion mutants of TacA to SVLVs in PC12 cells. A series of deletion mutations were made within the C-terminus of VAChT using the TacA construct as template. SVLV targeting of the mutants were determined by both immunofluorescent colocalization with synaptophysin, as well as presence in SVLV isolating glycerol velocity gradients. Symbol (+) indicates the targeting of TacA mutant to SVLVs. Symbol (--) indicates non-targeting to SVLVs. C) Immunofluorescent analysis of subcellular localization of key deletion mutants. PC12 cells transiently transfected were stained for mutant TacA (red) and synaptophysin (green). Bar= 20μm.

2.3.3 Dileucine containing motif is sufficient for SVLV targeting

As the results from our deletion analysis suggested that the dileucine containing motif in VAChT plays an essential role in SVLV targeting, we next examined the sufficiency of this motif to target the unrelated protein, Tac, to SVLVs. We therefore designed a construct, Tac8ADLM, in which the 10 a.a. (479-488) dileucine motif containing region of VAChT, preceded by an 8-alanine peptide, was fused to Tac (Figure 7A). It has been shown that the internalization machinery associated with dileucine motifs works most efficiently when this motif is at least eight amino acids away from the last transmembrane domain of a membrane protein (Bonifacino and Traub, 2003). Therefore, in order to avoid any possible disruption of internalization, we inserted an eight alanine peptide between Tac and the short dileucine containing motif of VAChT. Density gradient fractionation of PC12 cells transiently expressing Tac8ADLM (Figure 7B) showed the chimeras' targeting primarily to synaptophysin containing fractions. Further examination of this targeting using SV isolating velocity gradients showed consistent results. Tac8ADLM was enriched in SVLV fractions as marked by synaptophysin (Figure 7C). Together, these data strongly suggest that the dileucine containing motif of VAChT alone is able to target Tac to SVLVs in PC12 cells.

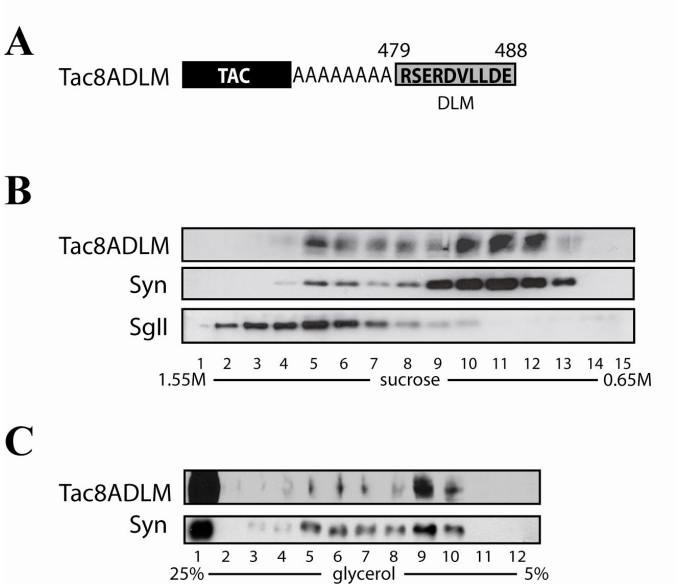

Figure 7. Dileucine containing motif is sufficient for SVLV targeting in PC12 cells.

A) Schematic diagram of Tac8ADLM. Eight alanines (8A), followed by the dileucine containing motif (DLM) of VAChT, were fused to the C-terminal end of Tac protein. B) Tac8ADLM targets to SVLV in PC12 cells using density gradient fractionation. PNS of PC12 cells transiently expressing Tac8ADLM were fractionated through sucrose density gradients. Tac8ADLM comigrated with synaptophysin containing fractions. C) Tac8ADLM targets to SVLV in PC12 cells using velocity sedimentation. Tac8ADLM comigrates primarily with synaptophysin containing fractions.

2.3.4 The dileucine containing motif serves as an internalization motif as well as a SVTM

The above results demonstrate that the VAChT dileucine containing motif is able to target Tac to SVLVs. The identified region contains a classical dileucine motif, E(XXX)LL, which has been extensively characterized for its role in internalization (Bonifacino and Traub, 2003; Tan et al.,

1998). Therefore, it was unclear whether the trafficking signal that mediates internalization is different than the one that mediates SVLV targeting. To address this, we generated a series of pair-wise alanine mutations within this region (Figure 8A) and analyzed their effects on internalization as well as SVLV targeting. To examine internalization, a semi-quantitative, ELISA based biotin conjugated system was used. The assay detected a fraction of internalization of TacA dialanine mutants over a 30 minute time period. Confirming the efficacy of our assay, wild type Tac protein remained primarily on the cell surface with only 25% internalization, whereas the majority (92%) of TacA was internalized. Truncation of TacA to TacA490 did not significantly alter internalization. Using pair-wise alanine mutations of this dileucine containing region, we showed that the mutation of LL-485/486-AA disrupted internalization dramatically (31% internalization compared to control) (Figure 8B). This result is consistent with previous studies that demonstrate the requirement for the leucine residues for efficient VAChT internalization (Bonifacino and Traub, 2003; Santos et al., 2001; Tan et al., 1998). Previously, the residues upstream of the leucines in many classical dileucine motifs have been indicated to regulate the efficiency of internalization. Not surprisingly, mutation of upstream residues DV 483/484 to AA in the VAChT C-terminus led to a significant decrease in efficiency of internalization over 30 minutes (Figure 8B). Mutation of residues 479/480 led to a small but significant decrease in internalization. Neither downstream dileucine flanking residues 487/488 nor residues 481/482 significantly contributed to the internalization of TacA, as mutation of these residues did not significantly alter the internalization of these mutant TacA proteins (Figure 8B).

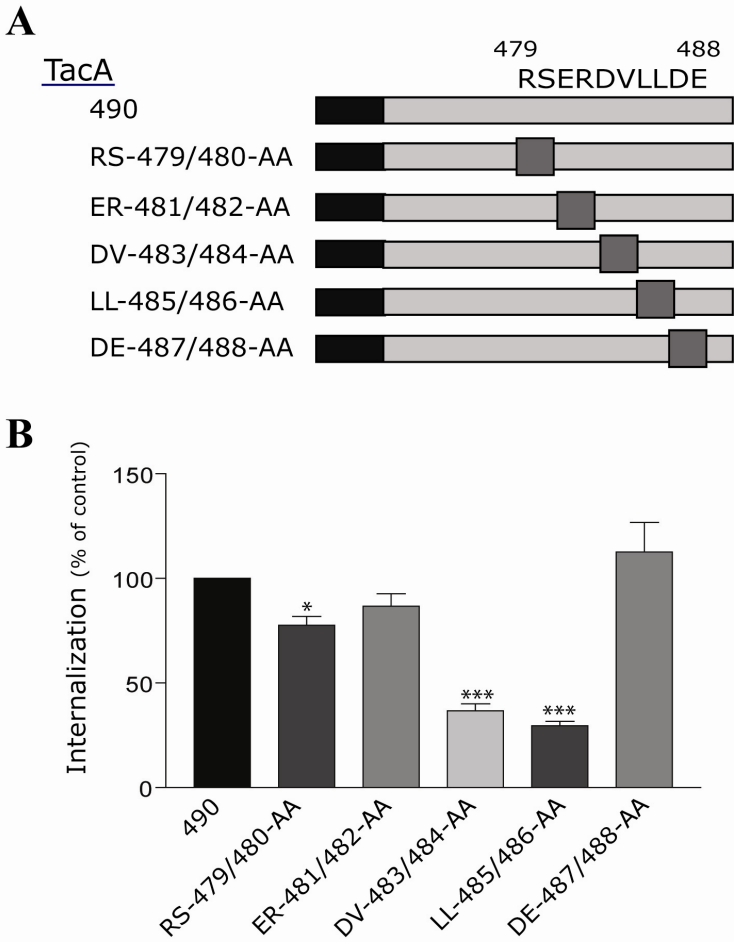

Figure 8. Dileucine containing motif is essential for the internalization of TacA.

A) Schematic diagram of dialanine scanning mutants of TacA. Pairwise alanine mutations (grey box) were made using TacA490 as the template. B) Mutation of leucines and neighboring residues reduces internalization of TacA. PC12 cells transiently transfected with TacA dialanine mutants were analyzed semi-quantitatively for their endocytosis for 30 minutes. Percent internalization of mutants was normalized to non-mutant TacA490 as control to determine effect on internalization (* $p<0.05$, ***$p<0.001$). Error bars depict standard deviation.

In parallel to the internalization assay, SVLV targeting of dialanine scanning mutants was analyzed by using confocal immunofluorescent imaging and density fractionation. As shown in figure 9A, the SVLV staining pattern of TacA was disrupted by mutation of the leucines (LL-485/486-AA) but not by mutation of other residues in the dileucine containing motif. Mutation of upstream residues DV-483/484-AA did not lead to significant accumulation of the chimera on the cell surface at steady state (Figure 9A), despite a decrease in its internalization at 30 minutes. This may suggest that while these residues decrease the efficiency of internalization, steady state internalization is not significantly affected as detected in our assay system.

Biochemical examination of dialanine mutants through density gradient fractionation confirmed our immunofluorescence data. As shown in figure 9B, peak fractions for all mutant TacA proteins, except the dileucine mutant, correspond with peaks of SVLV containing fractions. A shift in the peak of the dileucine mutant (LL-485/486-AA) towards heavier fractions, possibly containing endosomal and plasma membrane derived vesicles, suggests that this mutant does not target to SVLVs, presumably trapped at the cell surface as indicated through staining (Figure 9A). Together with both internalization and fluorescent staining, these results suggest that the dileucine containing region, identified as sufficient for SVLV targeting, does not harbor a separate SVTM. Rather, the dileucine motif may have dual properties for both internalization and SVLV targeting. Accordingly, the ability for the C-terminus of VAChT to direct trafficking toward SVLVs is influenced by its ability for internalization.

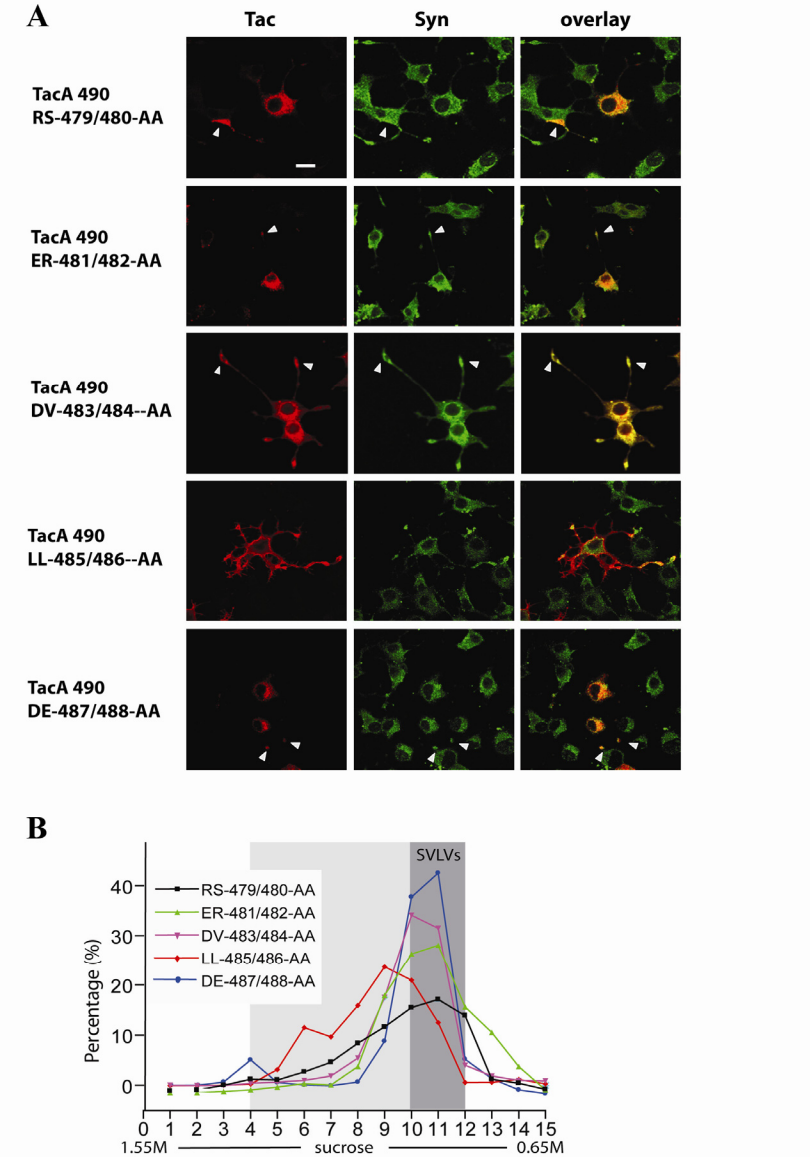

Figure 9 Dileucine containing motif of VAChT C-terminus serves as a SVTM.

A) SVLV targeting analysis of dialanine mutants as detected through immunofluorescent staining. Overexpressed TacA490 mutants in differentiated PC12 cells were visualized (red) and compared to staining patterns of synaptophysin (green). Only TacA490 LL-485/486-AA showed disrupted SVLV targeting, localizing preferentially to the cell surface. Arrows indicate the tips of the processes for colocalization of TacA variants and synaptophysin. Bar= 20µm. B) SVLV targeting analysis of dialanine mutants through density gradient analysis. PNS of transiently transfected PC12 cells were fractionated with sucrose gradients. Western blots for TacA490 mutant proteins were semiquantified and calculated as a fraction of total signal. Grey shading marks SVLV corresponding fractions.

2.3.5 Specificity of the dileucine containing motif for SVLV targeting

Our dialanine scanning analysis demonstrated that the SVLV targeting of TacA is limited only by mutation of the residues that interfered with internalization (leucine residues), indicating that the motif's role in SVLV targeting is not easily distinguishable from its previously defined role in internalization. We therefore sought to address the specificity of the VAChT dileucine containing motif as a SVTM. In order to address this, we examined additional Tac chimeras of several membrane proteins that contain other cytosolic internalization motifs. First, a chimera between Tac and the N-terminus of transferrin receptor (TacTfR), which contains a tyrosine internalization motif, was analyzed for its subcellular distribution. TacTfR was internalized from the plasma membrane but did not traffic to SVLVs as analyzed by density gradient (data not shown), supporting that the specificity of the VAChT dileucine containing motif as a SVTM extends beyond internalization. However, dialanine scanning mutagenesis of the VAChT dileucine containing motif suggested that none of the specific residues within this motif, other than the leucine residues, are required for the chimera's SV targeting (Figure 9). Therefore, it was unclear to what extent the exact motif in VAChT was specific as a SVTM and to what extent the dileucine motif could be generalized as SVTM. Thus, we tested the potential SV targeting of another classical dileucine motif containing protein, GLUT4. In PC12 cells GLUT4, regulated in part through a C-terminal dileucine internalization motif, R(XXX)LL, traffics to a unique vesicle population (Cope et al., 2000; Garippa et al., 1996; Herman et al., 1994; Thoidis and Kandror, 2001; Verhey et al., 1993). Examination of the steady state localization of TacGLUT revealed that the chimera, similarly to the full length protein, segregated to fractions slightly heavier than synaptophysin containing fractions in density gradients (Figure 10B). In order to ensure that TacGLUT did not comigrate with SVLVs, velocity gradient fractionation was performed to

separate SVLV light fractions with neighboring fractions. Exclusion from SVLVs was confirmed as TacGLUT was found only in the bottom fraction of the gradient (Figure 10C). Although both GLUT4 and VAChT chimera contain similar dileucine motifs, [R(TPS)LL vs. E(RDV)LL], their differential targeting suggests that the environment or nature of the residues of the dileucine motif may influence the specificity of this motif for SVLV targeting. Finally, we examined a closely related dileucine motif, E(XXX)[I/L]L, found in the similar environment of the C-terminus of VMAT2. VMAT2 was previously shown to have preferential targeting to LDCVs in PC12 cells, which is thought to be regulated by an acidic patch at the distal end of the C-terminus (Waites et al., 2001). The presence of this additional signaling motif also allowed us to examine how neighboring domain(s) influence the dileucine mediated targeting event. We therefore generated a chimera, TacM, in which the C-terminus of VMAT2 was fused to Tac protein (Figure 10A). In both transiently transfected and stable cell lines, as determined by sucrose and velocity gradients, TacM was found to traffic preferentially to LDCV fractions, but also to SVLV fractions (Figure 10D; velocity gradient not shown), suggesting the C-terminus of VMAT2 is not sufficient for its preferential LDCV targeting. To determine if the distal sequence of the acidic patch influences the function of C-terminus in targeting as indicated previously, we next constructed TacMs in which the acidic patch was deleted (Figure 10A). In contrast to the two peak distribution seen in the density gradient for TacM, the steady-state distribution of TacMs showed a single peak that co-migrated with synaptophysin containing fractions (Figure 10E). The trafficking of TacMs to SVLVs strongly suggests that the VMAT2 dileucine motif, when not influenced by neighboring signals, is also able to direct SVLV targeting, indicating that this motif might be generalized as a SVTM for several SV proteins.

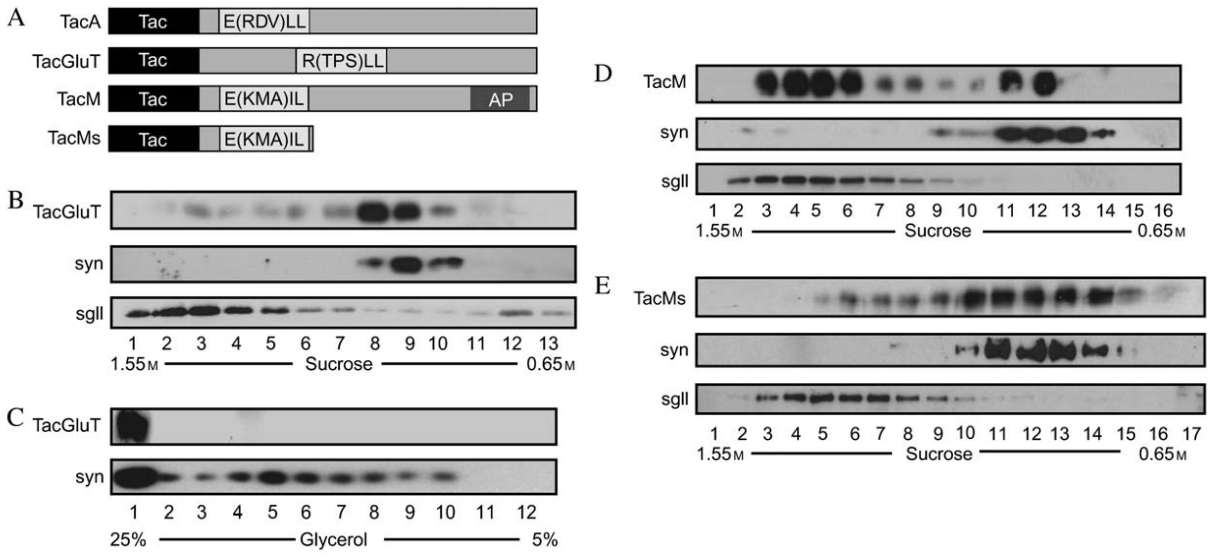

Figure 10. Vesicular targeting of membrane proteins that contain dileucine containing motifs.

A) Schematic diagram of constructs for TacA, TacGluT, TacM, and TacMs chimera. Dileucine motifs indicated in grey boxes. TacGluT and TacM contain the cytoplasmic C-terminal tail for GLUT4 and VMAT2, respectively. AP = acidic patch. TacMs contains the distal deletion mutation of TacM. B) TacGluT does not target to SVLVs in PC12 cells. PNS of PC12 cells transiently expressing TacGluT was fractionated through sucrose gradients. TacGluT does not comigrate with SVLVs containing fractions. C) TacGluT is excluded from SVLV containing fractions in glycerol gradients. D) TacM localizes to both LDCVs and SVLVs in PC12 cells in sucrose gradients. E) TacMs localizes to SVLVs but not LDCVs in PC12 cells in sucrose gradients. TacMs comigrates with SVLV fractions.

2.4 DISCUSSION

In this study, by using chimeras between an unrelated plasma membrane localized protein, Tac, and the C-terminus of VAChT, we have shown that the C-terminus of VAChT is necessary and sufficient for SVLV targeting in PC12 cells. Consistently, deletion analysis of this region demonstrates that a 10 a.a. dileucine containing motif is sufficient for this SVLV targeting.

Further examination of this motif has suggested that it contains dual signals indistinguishable for internalization and synaptic vesicle targeting. Through the use of additional control chimeras, we have shown that this signal is specific as a synaptic vesicle targeting motif (SVTM) and that it may be generalized for multiple SV proteins.

Our approach to investigate the SVLV trafficking signals of VAChT through the use of Tac chimeric proteins allowed us to determine the sufficiency of potential targeting signals within VAChT. This system permitted independent analysis of the ability of VAChT C-terminal cytosolic regions to traffic a non-related protein, Tac, to SVLVs. Previous studies of VAChT trafficking have used mutagenesis of full length VAChT or chimera between VAChT and $VMAT_2$. Due to the high sequence similarity between these two transporters [~65% among transmembrane regions (Roghani et al., 1994)], studies of this nature cannot exclude the potential role of the transmembrane domains or the luminal loop of the transporters in vesicular targeting. In addition to determining sufficiency, the Tac chimeric approach also allowed us to focus on internalization and sorting steps from the plasma membrane, which mimic SV recycling and biogenesis at neuronal terminals. Consistent with VNT chimeric studies (Tan et al., 1998), our results using Tac chimera in PC12 cells imply that neither the N-terminal domain nor the transmembrane domain regions of VAChT are required for SVLV trafficking at the terminal. However, our data does not address whether these regions are important in axonal targeting, an important additional sorting step for SV proteins in neurons (Prado and Prado, 2002). Our preliminary studies using TacA chimera in primary neurons show that the steady state distribution of the protein is not restricted to axons but extends to all neuronal processes (data not shown). This may suggest that in neurons the N-terminus or transmembrane regions of VAChT

may be required for the proper axonal targeting of the protein while the C-terminus may be sufficient to direct SV recycling at the terminal.

Our Tac chimeric analysis also first shows that the dileucine containing sequence (residues 479-488) within the VAChT C-terminus is sufficient as a SVTM in PC12 cells. Consistent with this, a similar region (residues 481 – 490) was previously indicated to be required in the targeting of full length VAChT in SN56 cells (Ferreira et al., 2005). On the other hand, our chimeric study was unable to confirm another report that a non-classical tyrosine motif (Y524-Y527) at the distal end of the C-terminus of VAChT serves as an alternative internalization motif or a SVLV trafficking role of a classical tyrosine motif (Kim and Hersh, 2004). Our systematic analysis of deletion mutants of these two tyrosine motifs failed to show any significant decrease in their internalization at 30 minutes or any alteration of their steady-state SVLV trafficking (Figures 6, 8). It is plausible to suggest that these previously identified motifs, while not required for SVLV targeting in our system, may play a role in regulating the efficiency of pathways of VAChT targeting. More sensitive approaches may help to address how they influence the dominant dileucine containing motif.

Further examination of the signaling properties of the dileucine motif demonstrated that this signal is specific as a SVTM. In agreement with our dialanine scanning results and analysis of additional Tac chimera, the specific residues within the VAChT dileucine containing motif are not essential for its SVLV targeting. However, TacGLUT containing a similar dileucine motif did not traffic to SVLVs suggesting that the overall environment of the motif, as well as factors such as proximity of the motif to the transmembrane domain, or accessibility of cytosolic machinery to the motif may determine the specificity of the dileucine containing motif as a SVTM. Furthermore, our data suggests that the dileucine containing motif can be influenced by

adjacent regions of the protein, such as the acidic patch (AP) in $VMAT_2$. One possible explanation is that this additional signaling motif may mask the SVTM and act as a dominant earlier sorting signal at the TGN. The other possibility is that this motif may promote the Golgi-endosomal trafficking during retrieval of $VMAT_2$ and thus enhance its overall targeting to secretory granules at TGN. When the acidic patch is deleted, the closely related dileucine motif of $VMAT_2$ was able to direct targeting to SVLVs, suggesting that this signal may be generalized to several SV proteins. Interestingly, the notion that the $VMAT_2$ C-terminus may contain signals for targeting to both LDCVs and SVLVs suggests some regulation of secretory vesicle protein trafficking between the two types of vesicles in neurons.

The potential for this type of dileucine motif to be a more general SVLV signal is further supported by several previous findings. In synaptotagmin 1, the cytoplasmic motif, E(VDA)ML, accounts for at least a portion of its SVLV trafficking in PC12 cells (Blagoveshchenskaya et al., 1999). Furthermore, the dileucine motif of Tyrosinase, E(KQP)LL is responsible for its targeting to SVLVs when exogenously expressed in PC12 cells (Blagoveshchenskaya et al., 1999). Interestingly, dileucine-like motifs have been identified in several other vesicular transport proteins such as VGLUT1 (**E**KCG**FV**) (Voglmaier et al., 2006). This recent report has suggested that this dileucine-like motif is critical for the internalization and trafficking of the transporter. This sequence is upstream of a polyproline motif that is responsible for interaction of VGLUT1 with endophilin 1, which may regulate this dileucine-like dependent trafficking (De Gois et al., 2006; Voglmaier et al., 2006).

The mechanisms of the dileucine mediated trafficking of synaptotagmin and tyrosinase are mediated by a fairly well characterized mechanism for SV biogenesis in PC12 cells (Faundez et al., 1998), Arf GTPase-AP3 directed endosomal budding (Blagoveshchenskaya et al., 1999).

However, AP-3 binding has not been able to be detected for VAChT (Kim and Hersh, 2004). Thus, VAChT targeting may be regulated through alternative cytosolic machinery. Accordingly, we have identified a novel functional interaction between the C-terminus of VAChT and SNX5, a lipid binding protein involved in the endosomal trafficking of membrane proteins (Colgan, et al., and manuscript in preparation. See Chapter 3). This suggests that VAChT may also traffic through an endosomal intermediate. The dual properties of the dileucine containing motif may regulate trafficking through an endosomal intermediate by serving first as an internalization signal at the plasma membrane and subsequently acting as a SVTM at an endosomal compartment. This sequential targeting model may rely on the chronological function of multiple regulatory sorting machineries or may take advantage of similar, but subcellularly compartmentalized machinery that govern different targeting events. Similar mechanisms are thought to occur in non-neuronal cell lines, where this type of dileucine motif can be recognized both at the plasma membrane as well as endosomal and lysosomal compartments (Bonifacino and Traub, 2003). Alternatively, the dileucine motif signal may mediate direct and selective internalization from the plasma membrane which has been suggested as a major pathway for recycling SVs in the nerve terminal (De Camilli and Takei, 1996). Nonetheless, the dual role of the dileucine-containing motif in internalization and SV targeting provides a molecular mechanism underlying the efficient recruitment of essential SV components during its biogenesis and recycling.

2.5 MATERIALS AND METHODS

2.5.1 Chemicals and antibodies

General chemicals used in this report were purchased from Sigma (St. Louis, MO) unless otherwise noted. The following antibodies were used in immunofluorescent staining: biotin conjugated mouse anti-CD25 (Affinity BioReagents, Golden, CO), polyclonal synaptophysin and secretogranin II (SYSY, Goettingen, Germany), secondary Cy3 conjugated goat anti-mouse and Alexa 488 conjugated goat anti-rabbit (Jackson Immunoresearch Lab, West Grove, PA). The following antibodies were used for Western blot: polyclonal IL-2R alpha (Santa Cruz Biotech., Santa Cruz, CA), monoclonal anti-IL2 Receptor (Covance, Princeton, NJ), polyclonal anti-synaptophysin and anti-secretogranin II (SYSY, Goettingen, Germany), secondary HRP conjugated goat anti-mouse and goat anti-rabbit (Pierce, Rockford, IL). Antibodies used for ELISA internalization assay are as follows: biotin conjugated mouse anti-CD25 (Affinity BioReagents, Golden, CO), polyclonal goat anti-mouse IgG (BD Pharmingen, San Diego, CA).

2.5.2 Plasmid construction and mutagenesis

Chimeric protein TacA was generated by PCR amplification of the C-terminus of VAChT (a.a. 465-530) with the introduction of Xba1 and Xho1 restriction sites flanking the region. PCR product was digested and subcloned into Tac/pcDNA 3.1 as described (Tan et al., 1998). C-terminus deletion mutant chimeras were constructed similarly by PCR amplification introducing an Xba1 site at the 5' region and a stop codon and Xho1 site at the 3' region. Digestion was followed by subcloning into Tac/pcDNA3.1 using Xba1 and Xho1. 8ATacDLM was constructed

using an extended 3' primer encoding and Xba1 site followed by 8 alanines and consensus motif for a.a. 479-483. PCR amplification introducing a stop codon and a Not1 site downstream of the dileucine region was followed by digestion and subcloning into Tac/pcDNA3.1 using Xba1 and Not1 restriction enzymes. Dialanine scanning point mutations were generated by using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as directed using TacA490/pcDNA3.1 as a backbone. TacGLUT was generated by PCR with oligos that isolated the C-terminus of human GLUT4 and introduced flanking Xba1 and Not1 restriction sites. PCR product was digested and subcloned into Tac/pcDNA 3.1 using Xba1 and Not1. TacM constructs were generated similarly to TacA with the exception that the C-terminus of VMAT2 was amplified by PCR and used for subcloning. TacMs was generated by introducing a stop codon after the dileucine containing region by QuickChange site directed mutagenesis using TacM/pcDNA 3.1 as a backbone. All constructs were confirmed by sequencing.

2.5.3 Cell culture and transfection

All cells were maintained in 5% CO_2 at 37ºC in medium containing penicillin and streptomycin unless otherwise noted. PC12 cells were maintained in DMEM (Invitrogen) with 10% Equine serum (Hyclone, Logan, UT), 5% Cosmic Calf serum (Hyclone, Logan, UT), and 2 mM L-Glutamine (Invitrogen). For immunofluorescent analysis cells were seeded on poly-D-lysine and Matrigel coated glass coverslips 24 hours before transfection. Cells on coverslips were then transfected using either LipofectAMINE 2000 (Invitrogen, San Diego, CA), or Superfect (Qaigen, Hilden, Germany) reagent according to the manufacturer's instructions. Twelve hours later cells were differentiated using 100 nM NGF for 24 hours before fixation with 4%

Paraformaldehyde. For biochemical assays, transfections were done using LipofectAMINE 2000. Transfected cells were incubated at 37ºC for 36 - 48 hours before harvest.

Stable lines of wild type Tac, TacA, TacA490 dialanine scanning mutants, and TacM were generated by further selection with G418 (500mg/ml). Positive transformants were screened by immunofluorescence staining and western blot analysis. At least three stable lines were tested for consistent subcellular localization of the heterogeneously expressed membrane proteins. All stable cell line derived results were also confirmed in transiently expressing independent PC12 cell lines.

2.5.4 Immunofluorescence and confocal microscopy

Immunofluorescent staining was performed as previously described (Liu et al., 2006). In brief, cells seeded on glass coverslips were fixed with 4% paraformaldehyde in PBS, pH 7.4. After fixation, cells were permeabilized and blocked for 30 min. in blocking buffer (BB, 2% BSA, 1% fish skin gelatin, and 0.02% saponin in PBS). Cells were then incubated with primary antibody in BB for 1 hour at room temperature. Coverslips were washed and incubated with the appropriate Alexa- or Cy3- conjugated secondary antibody for 1 hour at room temperature. Confocal images were acquired with a Fluoview 500 laser scanning confocal imaging system (Olympus, Tokyo, Japan) configured with a fluorescence microscope fitted with Pan Apo 60× and 100× oil objectives (Olympus). Confocal images were collected sequentially at 1024×1024 resolution to minimize bleed through of fluorescence between channels.

2.5.5 Fractionation analysis

PC12 cells stably expressing, or transiently transfected with plasmid constructs, were harvested in Buffer A (150 mM NaCl, 10 mM Hepes pH 7.4, 1 mM EGTA, 0.1mM MgCl2) with protease inhibitors. Cells were cracked by eight passes through a cell cracker (Clearance-0.02um). Post-nuclear supernatants were then loaded onto prepared density or velocity gradients and spun in a Beckman SW41 rotor. For sucrose density fractionation, sucrose gradients were prepared using a gradient mixer to form continuous gradients with sucrose concentrations from 0.65 M to 1.55 M and spun in SW41 rotor at 30,000 rpm for 8 hours at 4°C. (Beckman Instruments, Palo Alto, CA). Glycerol velocity fractionation was done through a glycerol gradient of 5% to 25% glycerol (V/V) and spun in SW41 rotor at 37,000 rpm for 1 hour at 4°C. Fractions from density gradients were collected from the bottom and velocity gradients collected from the top to minimize contamination. All gradients were numbered from heavy to light.

2.5.6 Western blot analysis

Proteins were detected in gradient fractions by immunoblotting as previously described (Chen et al., 2005). Equal amounts of gradient from each fraction were denatured in 3x SDS sample buffer (New England Biolab, Beverly, MA), and separated by electrophoresis through 10% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose (BA-85, Schleicher-Schuell Bioscience, Keene, NH) and TacA chimeric proteins, synaptophysin, secretogranin II, or other proteins were visualized by immunoblotting with appropriate antibodies in combination with enhanced chemiluminescence (Super-Signal West Pico, Pierce, Rockford, IL). Protein

immunoreactive signals were scanned and the intensity of bands was semi-quantified using the NIH Imaging program.

2.5.7 Internalization assay

Internalization assay was done as previously described (Tan et al., 1998). Briefly, PC12 cells transfected by electroporation with cDNA plasmids were harvested 36 hours later and resuspended with Serum Free Medium (DMEM, 20mM HEPES-KOH (pH7.2), 0.2% BSA). The cell suspension was incubated with biotinylated anti-CD-25 (Tac) monoclonal antibody at 4˚C for 2 hr. to label Tac chimeric proteins localized to the cell surface. After washing of unbound antibody, internalization was allowed for 30min by 37°C incubation. Internalization was stopped and the sample equally divided. In one fraction, cell surface labeled proteins were quenched through avidin incubation. Cells in both fractions were then solubilized and loaded in triplicate aliquots onto 96 well ELISA plate pre-coated with goat anti-mouse IgG and incubated overnight at 4˚C. Plates were washed of unbound biotin signal and developed using 3, 3', 5, 5' Tetramethylbenzidine (TMB) liquid substrate system for 30 minutes. Endpoint absorbance measurements were taken at 450nm using a BioRAD plate reader. Percent internalized was calculated as follows: (Ca-Va)/ (Ct-Vt) where Ca = internalized (avidin quenched) transfected cells; Va = internalized (avidin quenched) vector transfected cells; Ct = total (unquenched) transfected cells; Vt = total (unquenched) vector transfected cells. Standard curves were run with each experiment to ensure inclusion within the linear range of detection. Mutants were normalized to control TacA490 constructs to determined percent change in internalization and to normalize across experiments.

3.0 SORTING NEXIN 5 REGULATES THE SYNAPTIC VESICLE SPECIFIC TARGETING OF VESICULAR ACETYLCHOLINE TRANSPORTER

3.1 ABSRACT

Packaging of neurotransmitter into small synaptic vesicles or large dense core vesicles is determined by vesicle-specific targeting of vesicular neurotransmitter transporters (VNTs). Their regulated targeting is mediated through interaction of sorting motifs with cytosolic machinery, however, machinery that regulates the vesicle specific trafficking of VNTs remains to be identified. Here we identify that sorting nexin 5 regulates the specific trafficking of vesicular acetylcholine transporter, responsible for packaging acetylcholine into synaptic vesicles. Disruption of sorting nexin 5 function leads to mislocalization of vesicular acetylcholine transporter to large dense core vesicles, suggesting a potential regulatory mechanism for membrane trafficking between secretory vesicles.

3.2 RESULTS

Information transfer in the nervous system is mediated through the regulated release of neurotransmitter (NT) from vesicular stores including clear synaptic vesicles (SVs) and large dense-core vesicles (LDCVs). The distinct release properties of these secretory vesicles, including calcium sensitivity, speed, and synchrony, determine characteristics of transmission (Edwards, 1998). Specific packaging of NT into either SVs or LDCVs is determined by the presence of vesicular neurotransmitter transporters (VNTs), whose localization to these secretory vesicles is highly regulated. Therefore, in order to understand the determinants of vesicle-specific NT packaging, we sought to identify machinery that regulates the trafficking of VNTs.

Vesicular Acetylcholine Transporter (VAChT) has been shown to localize preferentially to SVs, which determines the fast, synchronous release of Acetylcholine (Ach) in both neurons and neuroendocrine cell lines. We have previously shown that a synaptic vesicle targeting motif in the C-terminal tail of VAChT is sufficient for this SV-specific targeting in PC12 cells, a neuroendocrine cell line (Colgan et al., 2007). Therefore, in order to identify cytosolic machinery that regulates this trafficking, we isolated the VAChT C-terminus in a fusion protein with an unrelated plasma membrane protein, Tac. The chimera, TacA, efficiently traffics to SVs (Colgan et al., 2007). Therefore, we used the SV-specific trafficking of TacA as a tool to characterize interacting proteins that regulate SV trafficking.

3.2.1 Sorting Nexin 5 associates with VAChT

Using the C-terminus of VAChT as bait in a yeast-two-hybrid screen, we identified sorting nexin 5 (SNX5) as a potential interacting protein that could regulate trafficking to synaptic vesicles.

SNX5 is a member of the sorting nexin family, characterized structurally by a homologous membrane binding Phox (PX) domain, and functionally, by regulation of membrane protein trafficking (Worby and Dixon, 2002). In addition to a PX domain, SNX5 contains a curvature sensing BAR domain. Functionally, the role of SNX5 has not been clearly defined although it has been suggested to regulate endosomal or Golgi trafficking in non-neuronal cells (Liu et al., 2006; Wassmer et al., 2007). The potential interaction of SNX5 with VAChT was investigated through binding assays. Pull down experiments suggested that the two proteins can bind and that this binding depends on both the C-terminus of VAChT and the BAR domain of SNX5 (Figure 11B and Figure S2).

In order to characterize SNX5 in the nervous system, we analyzed its tissue distribution through Northern and Western blots of rat tissue. SNX5 RNA message showed a relatively widespread tissue distribution (data not shown) in agreement with previously published results (Otsuki et al., 1999). However the protein expression of SNX5 was highly enriched in tissue from brain and testis (Figure 11C). Subcellular distribution analysis revealed that SNX5 is present in both soluble and lipid bound fractions in Hela cells (data not shown), characteristic of the SNX proteins' ability to transiently associate with membrane. In agreement with proteomic identification of SNX5 as a SV protein (Takamori et al., 2006), SNX5 was present in the SV-enriched LP2 subcellular fraction of rat brain homogenate (data not shown) and partially colocalized with markers of SVs in PC12 cells (Figure 11C). Thus, SNX5 is enriched in the brain and able to associate with SVs, further indicating a potential role for SNX5 in regulating SV trafficking of VAChT.

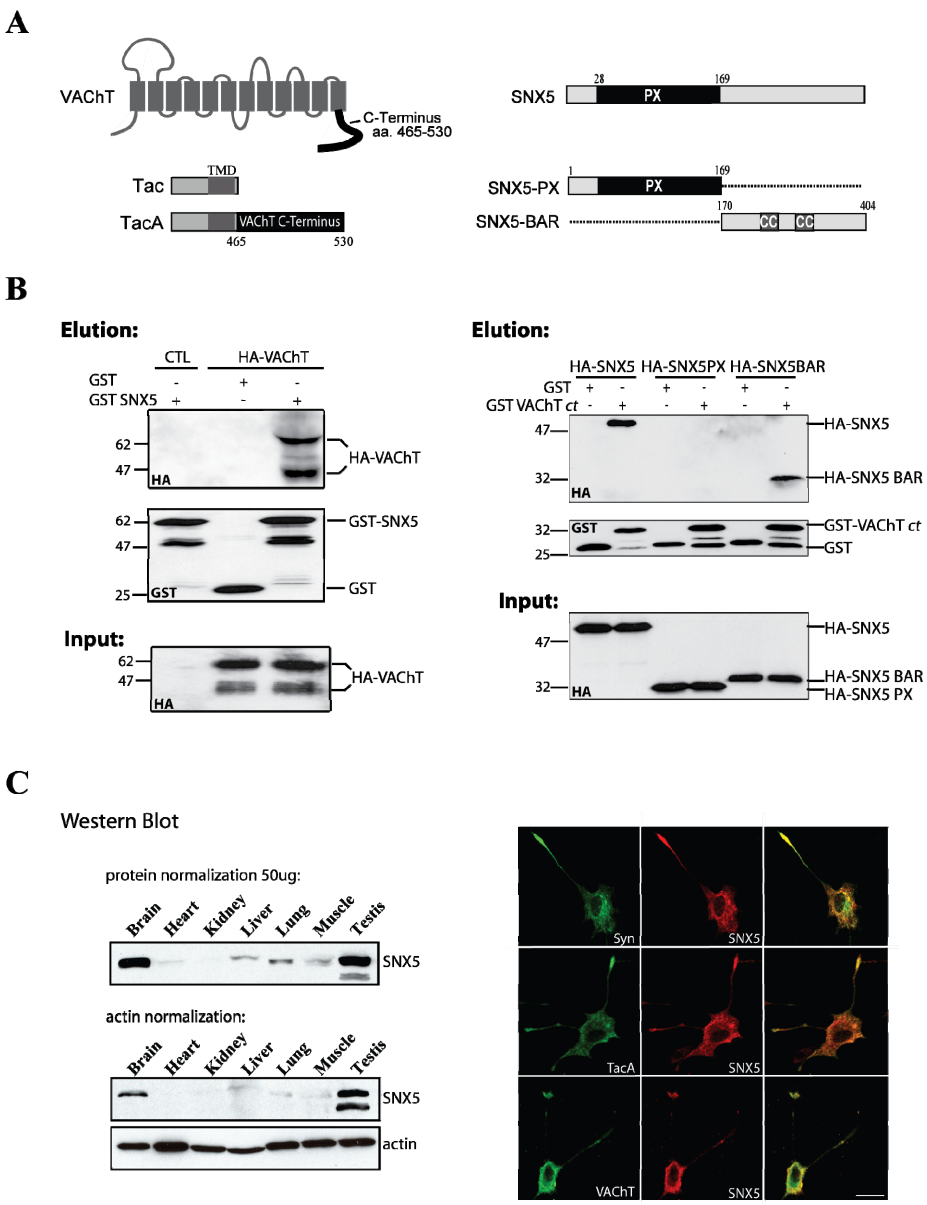

Figure 11. SNX5 associates with VAChT

A) C-Terminus of VAChT (black) was isolated and fused to Tac protein (TacA). SNX5 Phox domain (PX) and SNX5 BAR domain (CC/CC) are indicated. B) HEK293 cell lysates, transiently transfected with HA-VAChT, HA-SNX5, HA-SNX5 PX, or HA-SNX5BAR were incubated with glutathione-sepharose immobilized GST-SNX5 or GST-VAChT C-terminus as indicated. Bound protein was eluted and detected by Western blot. C) Tissue distribution of SNX5 protein in rat homogenate. SNX5 was detected through Western blot by specific antibody. D) Immunofluorescence imaging of endogenous SNX5 (red) in differentiated PC12 cells. Markers of synaptic vesicles including synaptophysin, TacA, and VAChT (green) were also detected.

3.2.2 SNX5 regulates SV Trafficking

Analysis of the potential function of SNX5 in regulating the SV specific trafficking of VAChT was studied by altering SNX5 levels in PC12 cells stably expressing the chimeric protein TacA. The expression level and localization of TacA were monitored to detect resulting changes in SV protein trafficking. SNX5 levels were altered through three methods, overexpression of SNX5, overexpression of dominant-negative truncated SNX5 constructs (SNX5BAR and SNX5PX), and siRNA mediated knockdown of endogenous SNX5. Overexpression of SNX5 did not significantly alter the expression level or localization of TacA. This may reflect the efficiency of the interaction between SNX5 and the VAChT C-terminus. Furthermore, it suggests that overexpression of SNX5 does not grossly disrupt cellular trafficking pathways. Overexpression of SNX5 mutants or knockdown of endogenous SNX5, however, led to a significant and dramatic (~40%) loss in SV targeting of TacA (Figure 12A, 12B) without an alteration of protein level. This effect, underrepresented in the data due to the ~ 60% maximum transfection efficiency in our system, suggests that SNX5 regulates trafficking of VAChT to SVs. The regulation of this SV trafficking appears to have some specificity, as the trafficking of another SV localized protein, synaptophysin (p38), was not altered (Figure 12A, B).

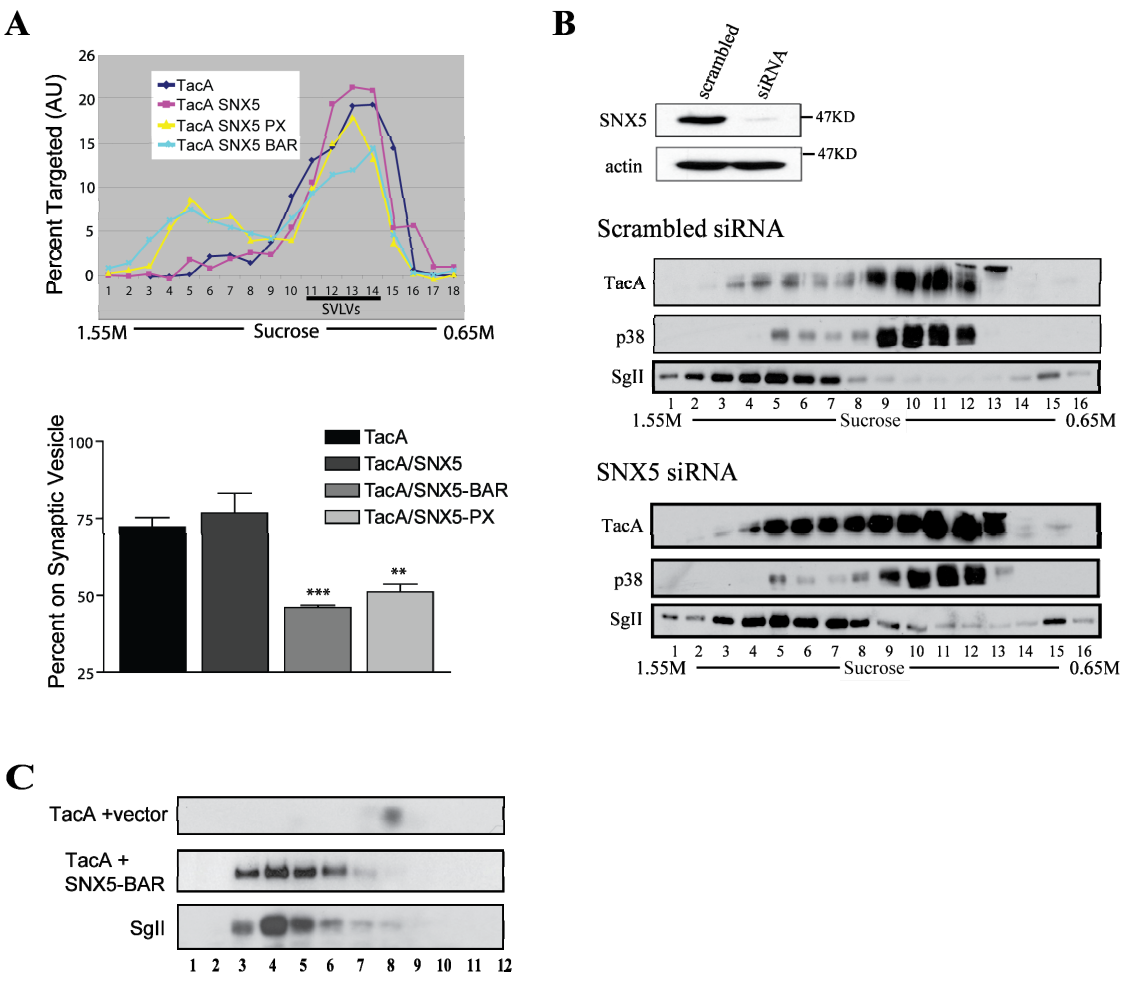

Figure 12. SNX5 regulates trafficking of VAChT to SVs

A) Stable PC12 cell lines expressing TacA were transfected with SNX5 or SNX5 mutants. Postnuclear supernatants were fractionated through an equilibrium sucrose gradient and probed for TacA and the SV marker, synaptophysin, through western blotting. Fractions 11-14 correspond to synaptophysin enriched fractions. Cosedimentation of TacA with SVLV marker synaptophysin was quantified. *** p=.0004 **p=.0035 B) Stable TacA expressing PC12 cells were transiently transfected with specific SNX5 siRNA or scrambled siRNA for 72h. Knockdown efficiency was examined by Western blot. Post nuclear supernatants of siRNA transfected PC12 cells were fractionated through sucrose gradients. Collected fractions were probed for TacA, synaptophysin (p38), or secretogranin II (SgII). C) Stable TacA expressing PC12 cells were transiently transfected with vector or with SNX5BAR. PNS was collected and run through a two-step gradient to isolate dense-core vesicles. Collected dense-core vesicle enriched fractions were probed for the presence of TacA.

As disruption of SNX5 decreased the SV localization of TacA without altering its expression level, we next determined the localization of the mistargeted protein. Using density gradient subcellular fractionation, we showed that TacA was shifted to fractions containing heavier organelles including LDCVs, endosomes, plasma membrane, or Golgi (Figure 12A, B). Using markers for these organelles, SGII, TfR, Na/K ATPase and TGN38 respectively, our data suggested that disruption of SNX5 lead to an accumulation of TacA on LDCVs (Figure 12B, Figure S2). However, density gradient fractionation alone is not sufficient to separate these heavier organelles from one another. In order to determine if SNX5 disruption led to an accumulation on LDCVs, we isolated LDCVs using a two-step gradient and examined the presence of TacA before and after disruption of SNX5 function (Tooze and Stinchcombe, 1992). Overexpression of SNX5 mutants led to a dramatic increase of TacA on LDCVs (Figure 12C) without altering its overall expression level. Thus, disruption of SNX5 function altered targeting of TacA from SVs to LDCVs.

The results of this work identify SNX5 as a novel regulator of the SV specific trafficking of VAChT. Further study is required to determine the location and mechanism of SNX5 regulation of VAChT trafficking, as well as the physiological relevance of this regulation in the nervous system. However, the identification of machinery for the vesicle specific trafficking of VAChT suggests that regulation of VNT trafficking may serve as a potential mechanism in defining the packaging of NT and thus, the properties of synaptic transmission.

4.0 COUPLING OF VESICULAR TRANSPORT AND SOMATIC RELEASE IN SEROTONIN NEURONS

4.1 ABSTRACT

Although neurotransmission relies on the packaging of transmitters into vesicles, it has not been possible to directly monitor the function of vesicular transporters as they support release in living neurons. Here, a pH-sensitive, fluorescent serotonin analog is visualized with two-photon microscopy to study vesicular monoamine transporter (VMAT) activity during somatic release in dorsal raphe nucleus serotonin neurons. Following uptake by the serotonin transporter and packaging by VMAT, glutamate receptor activation evoked somatic vesicular release of the fluorescent monoamine. Release was accompanied by VMAT activity, which redistributed monoamine from the nucleus, a compartment not previously implicated in neurotransmission. Measurements of vesicular transport and release allowed for the contribution of activity dependent packaging to somatic release to be assessed. While some monoamine packaged at rest was held in reserve, monoamine packaged during stimulation was released efficiently, suggesting a coupling between activity-dependent vesicular transport and somatic release.

4.2 INTRODUCTION

Neurotransmission requires vesicular transporters (VNTs), which package neurotransmitters into vesicles, and the exocytosis/endocytosis vesicle cycle, which supports release of packaged content. Although the vesicle cycle can be assayed in single neurons, it has not been possible to dynamically monitor packaging. For example, pH-sensitive, fluorescent secretory vesicle proteins can be used to track synaptic vesicle exocytosis and endocytosis based on the luminal acidic pH of synaptic vesicles (Burrone et al., 2006). However, this approach provides insight into vesicle cycling without detecting vesicle content (i.e., signals are identical for filled and empty vesicles). This limitation precludes detection of vesicular transporter activity, and likely accounts for reported mismatches between measurements of vesicle cycling and transmitter release (Ertunc et al., 2007; Tabares et al., 2007). Potentially, vesicular transport could be assayed by imaging transmitter dynamics directly. In fact, serotonin (5-hydroxytryptamine, 5-HT) is detected with three-photon microscopy, but vesicular content has only been detected in large granules and vesicle clusters. Thus far, transmitter in disperse, small vesicles and transport of 5-HT from the cytoplasm into vesicles has not been observed with this approach (Kaushalya et al., 2008a; Kaushalya et al., 2008b; Maiti et al., 1997). Thus, current methods cannot directly monitor vesicular transporter activity during stimulated release.

The complementary advantages of imaging pH-sensitive vesicle markers and vesicle content suggest a new possibility: imaging a fluorescent, pH-sensitive neurotransmitter analog. Such an analog would have to be recognized by native transporters and released in response to activity. Furthermore, in contrast to serotonin[4], the signal from the analog would change when transported from the neutral cytoplasm into acidic vesicles and allow for quantification of vesicular content even when individual vesicles cannot be resolved. A possible candidate for

this novel approach is the serotonin analog 5,7-dihydroxytryptamine (dHT). This monoamine absorbs near-UV light in a pH-dependent manner and emits visible light (Schlossberger, 1978; Vaney, 1986). It is a substrate of the serotonin transporter (SERT) and thus accumulates in serotonin neurons (Bjorklund et al., 1974). Furthermore, within hours of injection into animals, dHT is detected in serotonin neuron vesicles, raising the possibility that it is a substrate of the vesicular monoamine transporter (VMAT) (Gershon and Sherman, 1982). Prolonged exposure to dHT is toxic, but this effect is inhibited by preventing its chemical and enzymatic oxidation (Bjorklund et al., 1975; Silva et al., 1988). Indeed, acute dHT uptake identifies viable monoaminergic neurons without altering neuronal morphology or electrical properties (Hahn et al., 2006; Hahn et al., 2003; Silva et al., 1988). Therefore, imaging dHT might provide an approach to study vesicular transport during release in serotonin neurons.

Serotonin neurons located in the dorsal raphe nucleus (DR) project widely throughout the brain to control mood and behavior. Reduction of serotonin release, which can occur as a result of decreased VMAT activity, is linked to psychiatric disorders, while increasing serotonin levels with SSRIs (selective serotonin reuptake inhibitors) relieves depression (Fukui et al., 2007). In addition to release in projection areas, vesicular serotonin release occurs in the DR from the cell body and dendrites (de Kock et al., 2006) and can regulate the activity of serotonin release from terminals. This regulation is mediated by activation of inhibitory $5HT_{1A}$ autoreceptors that decrease neuronal firing rate, and is relevant to the delay in therapeutic efficacy of SSRI antidepressant drugs (Blier et al., 1998). Despite the importance of local serotonin release in the DR, little is known about the mechanisms and regulation of this somatic vesicular release.

Here, a new optical approach, two-photon imaging of a pH-sensitive, serotonin analog is developed to study vesicular transport and release in serotonin neurons of the DR. First, the

method is established with cultured cells and brain slice. Then regulation of somatic release in the DR is studied. Finally, intracellular monoamine dynamics are monitored to explore the contributions of vesicular transport at rest and during activity to release.

4.3 RESULTS

4.3.1 Two-photon excitation and pH sensitivity of dHT

The additional hydroxyl group of the serotonin analog dHT (Figure 13a) shifts its fluorescence emission into the visible range and renders broad pH dependence to its absorption (Schlossberger, 1978). To test whether dHT fluorescence is affected by the acidity found inside secretory vesicles, images of 500 μM solutions buffered to pH 5.5 (the pH inside secretory vesicles) and pH 7.4 were collected. Fluorescence was two-fold greater at pH 7.4 than at pH 5.5 (Figure 13b). Thus, unlike serotonin (5-HT) (Maiti et al., 1997), dHT fluorescence should be affected markedly by vesicular pH, a property that can be used to distinguish between vesicular and extra-vesicular monoamine.

dHT fluorescence is evoked by two-photon excitation. A solution of dHT in ascorbate was exposed to increasing wavelengths of infrared light and emitted fluorescence was collected as described in the Methods. Mean dHT fluorescence, after subtraction of the ascorbate signal, was maximal around 720 nm (Figure 13c), which is twice the wavelength for maximal single-photon excitation (Vaney, 1986). Fluorescence, excited at 725 nm, depended on the square of the excitation power. This is demonstrated by the slope (2.0 ± 0.01) in a log(fluorescence) versus log(power) plot (Figure 13d). Therefore, fluorescence was mediated by absorption of two

photons. Thus, the advantages inherent in two-photon microscopy, including thin optical sectioning with minimal photodamage and deep penetration into brain tissue (Svoboda and Yasuda, 2006; Williams et al., 1994), can be applied to dHT.

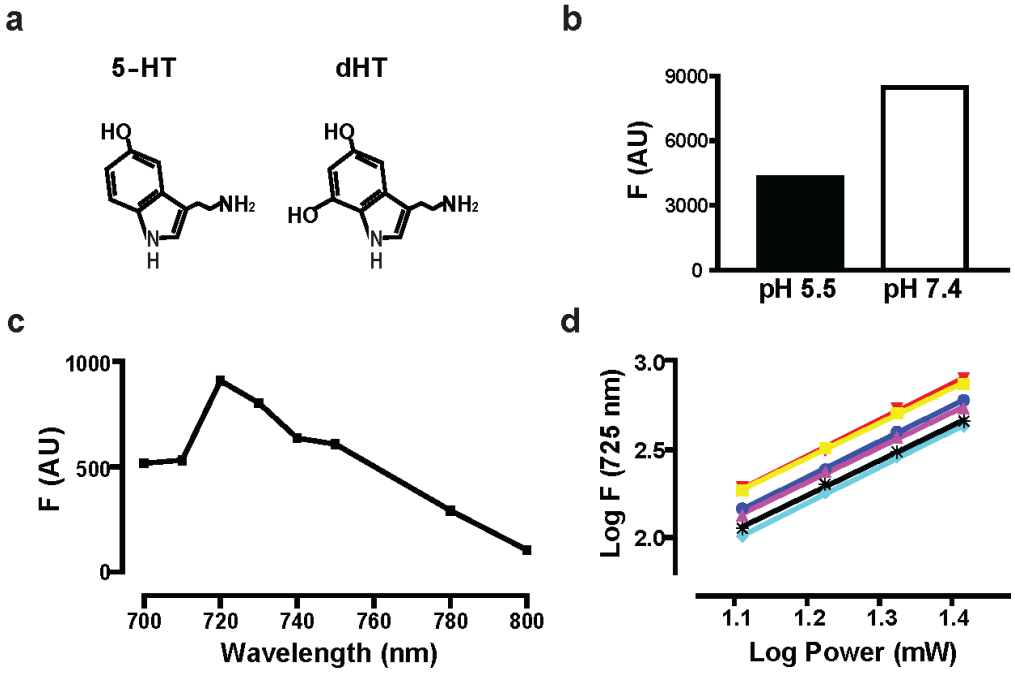

Figure 13. pH and two-photon characteristics of dHT.

(a) Structures of serotonin (5-HT) and dHT. (b) dHT fluorescence (F) is pH dependent. Images of pH 5.5 and pH 7.4 buffered dHT solutions were collected and background subtracted with control buffered solutions. (c) Empirical two-photon excitation spectrum of dHT. 5 mM dHT in 142 mM ascorbate was excited at indicated wavelengths on an upright, two-photon microscope. Collected fluorescence was background subtracted with signals from ascorbate. Note that this plot does not correct for the increase in laser output associated with increasing wavelength. (d) Two-photon absorption of dHT. Dorsal raphe slices loaded with dHT (see Methods) were excited with increasing levels of power measured at the specimen. The logarithms of the background-subtracted, mean fluorescence values of cell bodies were plotted versus the logarithms of excitation power. Symbols represent collected data and colored lines represent linear regression from individual cells (n=6). Average slope = 2.0 ± 0.01.

4.3.2 Cellular uptake and vesicular packaging of dHT by SERT and VMAT

To demonstrate specific uptake of dHT, PC12 cells transfected with SERT and GFP were incubated with 20 μM dHT. The antioxidant ascorbate and the monoamine oxidase inhibitor pargyline were also present to inhibit oxidation of dHT. Cells expressing SERT, but not a control vector, accumulated dHT within an hour (Figure 14). This accumulation was inhibited by preincubation of cells with the specific SERT inhibitor fluoxetine (Figure 14). Thus, SERT-mediated dHT uptake into cells was detected.

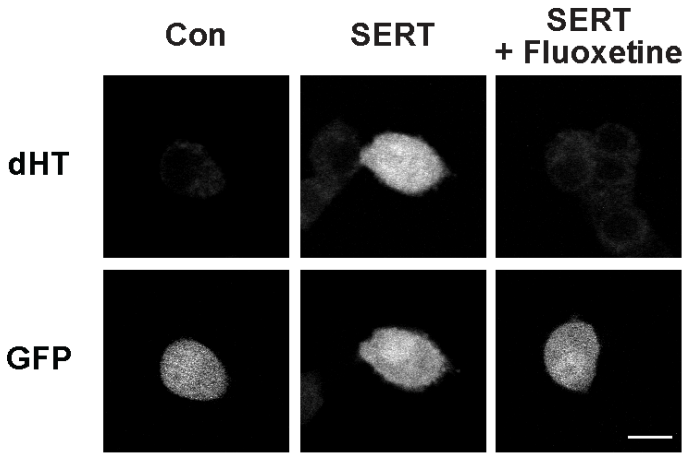

Figure 14. SERT-mediated dHT loading detected by two-photon microscopy.

Upper row: Two-photon dHT fluorescence images of representative undifferentiated PC12 cells transfected with control (Con) or SERT DNA and incubated for 1 h with dHT or a combination of dHT and fluoxetine. Lower row: Confocal single photon fluorescence of GFP was used to identify transfected PC12 cells. Scale bar = 10 μm.

Several experiments demonstrated that dHT is packaged by VMAT into secretory vesicles. First, SERT-transfected, NGF-differentiated PC12 cells were incubated overnight in dHT, washed for 1 hour and then imaged. In contrast to the distributed fluorescence pattern produced with 1 hour incubation, this longer protocol resulted in fluorescence localized to growth cones, the location of secretory vesicles in PC12 cells (Figure 15a). Second, application of 1 μM monensin, a cation ionophore that neutralizes the acidic pH in PC12 cell secretory vesicles (Han et al., 1999a), led to a two-fold increase in fluorescence in the growth cone (Figure 15b). The sensitivity to collapse of the vesicular pH gradient, along with the pH-sensitive fluorescence of dHT (Figure 13b), is consistent with efficient packaging of dHT into secretory vesicles. Third, incubation of SERT-transfected cells with 100 nM reserpine, a specific VMAT inhibitor, before and during the dHT incubation prevented the preferential accumulation of fluorescence in growth cones (Figure 15c, top). This was evident by the change in the ratio of fluorescence in growth cones to cell bodies (F_{GC}: F_{CB}) induced by reserpine (Figure 15c, bottom). Finally, induction of exocytosis by K^+-induced depolarization for 10 minutes evoked a 50 ± 3% loss of dHT fluorescence. In PC12 cells, VMAT-containing secretory vesicles also contain neuropeptides. Therefore, to confirm that this response is consistent with vesicular release, PC12 cells that express a GFP-tagged neuropeptide (ANF-GFP) (Burke et al., 1997; Han et al., 1999b) were transfected with SERT and loaded with dHT overnight. The fluorescence signals from dHT and the secretory vesicle marker colocalized in growth cones (Figure 15d, top). Furthermore, depolarization induced decreases in dHT and ANF-GFP fluorescence signals that were similar in magnitude and kinetics (Figure 15d, bottom). Thus, dHT fluorescence loss paralleled release of secretory vesicle content. Together, four independent criteria show that VMAT packages dHT into acidic, release competent secretory vesicles.

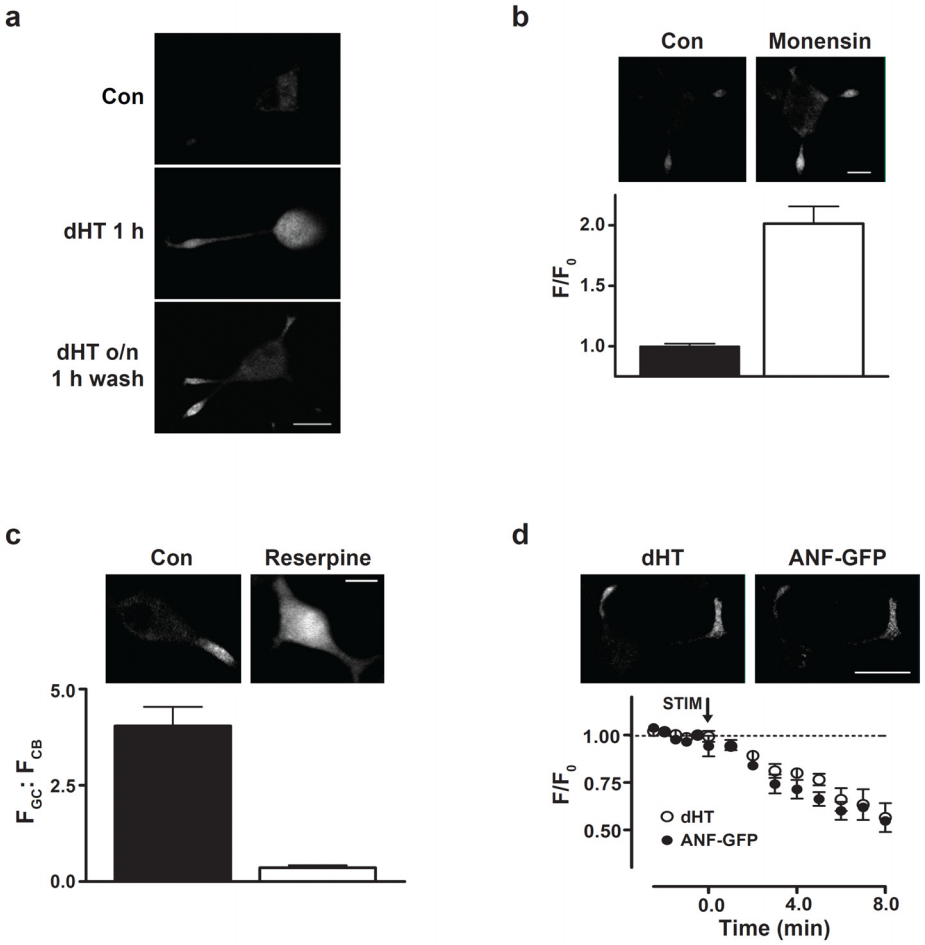

Figure 15. VMAT-mediated loading of dHT into release competent secretory vesicles.

(a) Representative two-photon images of NGF-differentiated PC12 cells loaded with control solution (Con) or dHT for 1 h or overnight (o/n) as indicated. O/n loading was followed by a 1 h wash. Scale bar = 10 µm. **(b)** dHT fluorescence doubles upon collapse of vesicular pH gradient. Upper: Representative images of differentiated PC12 cells before (Con) and 5 mins after application of 1 µM monensin. Scale bar = 10 µm. Lower: Quantification of monensin-induced changes in dHT fluorescence in growth cones (n=4). **(c)** Reserpine disrupts localized accumulation of dHT. Upper: Representative images of differentiated PC12 cells loaded o/n with dHT (Con) or 100 nM reserpine and dHT. Scale bar = 10 µm. Lower: Ratio of fluorescence in growth cone to fluorescence in cell body (F_{GC}: F_{CB}). ($n \geq 3$). **(d)** Parallel release of a neuropeptide and dHT. PC12 cells stably expressing the secretory granule cargo ANF-GFP were transfected with SERT and then loaded with dHT o/n. Upper: Colocalization of dHT and GFP epifluorescence in growth cones. Scale bar = 20 µm. Lower: K^+-induced depolarization (STIM) induces parallel decreases in dHT and ANF-GFP signals from growth cones (n = 6).

4.3.3 Two-photon imaging of somatic vesicular release in the raphe nucleus

Having established the use of dHT in a cell line, the applicability of two-photon dHT imaging was explored in dorsal raphe nucleus (DR) neurons. Coronal, DR-containing brain slices from p14-p21 rats were incubated in dHT, ascorbate and pargyline for 3 hours before imaging. This duration was chosen because brain slices are not typically viable overnight and previous studies indicated that dHT is detected in synaptic vesicles within 2-4 hours(Gershon and Sherman, 1982). Stacks of two-photon images showed that dHT fluorescence accumulated in neuronal cell bodies and processes of the DR with morphology expected for serotonin neurons (Figure 16a). However, fluorescence was never seen in slices incubated in 10 μM fluoxetine, a SERT inhibitor, before and during dHT loading (data not shown). This confirms that dHT accumulates through SERT into serotonin neurons in the slice. Furthermore, the requirement for dHT uptake and the two-photon excitation seen in dHT-loaded brain slices (Figure 13d) exclude that fluorescence derives from three-photon excitation of endogenous serotonin. Interestingly, the dHT signal was not punctate, suggesting that dHT might be present both in the cytoplasm and vesicles, as has been previously concluded (Balaji et al., 2005).

To examine the effect of stimulation, somatic fluorescence was compared from stacks of images through DR neurons acquired before and 1 minute after bath application of control solution (Con) or 10 μM AMPA, a glutamate receptor agonist (Figure 16b). AMPA, but not the control solution, induced a drop in the dHT signal. This fluorescence change ($\Delta F = 1-(F/F_o)$) required extracellular Ca^{2+} (Figure 16c). Furthermore, the AMPA response was inhibited by incubating slices in 50 nM reserpine during dHT exposure to prevent VMAT-mediated loading of vesicles (Figure 16c). Thus, AMPA evokes Ca^{2+}-dependent, vesicular dHT release from DR serotonin neuron cell bodies.

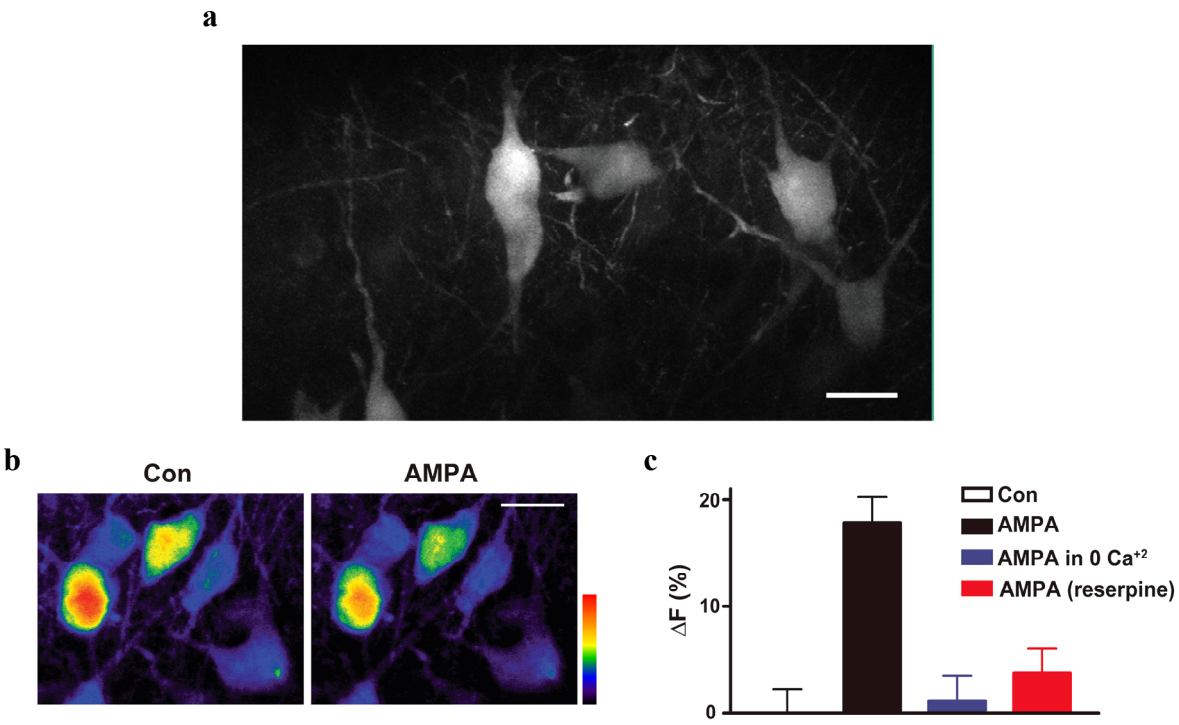

Figure 16. AMPA-induced somatic vesicular release from DR serotonin neurons.

(a) Maximum Z-projection of 16 consecutive, 2.5 µm-spaced two-photon images through the DR of rat brain slice loaded with dHT for 3 h. Scale bar = 20 µm. (b) Pseudo-colored summed Z-projection of stack of 20 consecutive, 2.5 µm-spaced images before (Con) and after 1 min of stimulation with 10 µM AMPA. Scale bar = 20 µM. (c) Quantification of the decrease in somatic fluorescence from dHT-loaded slices upon 1 min incubation with control solution (□), AMPA (■), or AMPA in zero calcium (■) as indicated. In addition, AMPA-induced fluorescence responses are show for slices loaded in the presence of reserpine (■). n ≥ 16 for each condition.

4.3.4 Autoreceptor-mediated inhibition of somatic release in the presence of an antidepressant

Treatment of depression with SSRIs shows a delayed onset of therapeutic effect, which is thought to involve activation of inhibitory $5HT_{1A}$ autoreceptors. Indeed, acute application of

SSRIs in the DR leads to an autoreceptor-mediated decrease in serotonin release (Adell and Artigas, 1991). However, the involvement of somatic release in this response is not known. To test whether this inhibition of release occurs at the serotonin neuron cell body, the SSRI fluoxetine was applied for 5 minutes before and during stimulation with AMPA. This treatment attenuated the AMPA-evoked somatic decrease in dHT fluorescence (Figure 17a). To determine whether the inhibitory effect of fluoxetine was mediated by $5HT_{1A}$ autoreceptors, the experiment was repeated in the presence of the selective $5HT_{1A}$ receptor antagonist WAY 100635. Bath application of 2 μM WAY 100635 did not alter resting fluorescence or AMPA-induced release. However, the autoreceptor antagonist blocked the fluoxetine-induced attenuation of release (Figure 17b). Thus, inhibition of somatic release by fluoxetine is mediated by activation of 5-HT_{1A} autoreceptors.

Several conclusions can be drawn from these data. First, because application of the 5-HT_{1A} receptor antagonist did not increase AMPA-evoked release, inhibitory autoreceptors do not affect responses to AMPA in the absence of fluoxetine. Second, fluoxetine had no effect on AMPA-induced release independent of 5-HT_{1A} receptors. Therefore, SERT does not mediate somatic release induced by AMPA stimulation. This confirms the finding that all detected release is vesicular (Figure 16c). Third, the inhibition of release in response to fluoxetine, through activation of the G-protein coupled autoreceptor, demonstrates the viability of the preparation and the fidelity of two-photon dHT imaging. Finally, autoreceptor-mediated, SSRI-induced inhibition of release occurs at the serotonin neuron cell body in the DR.

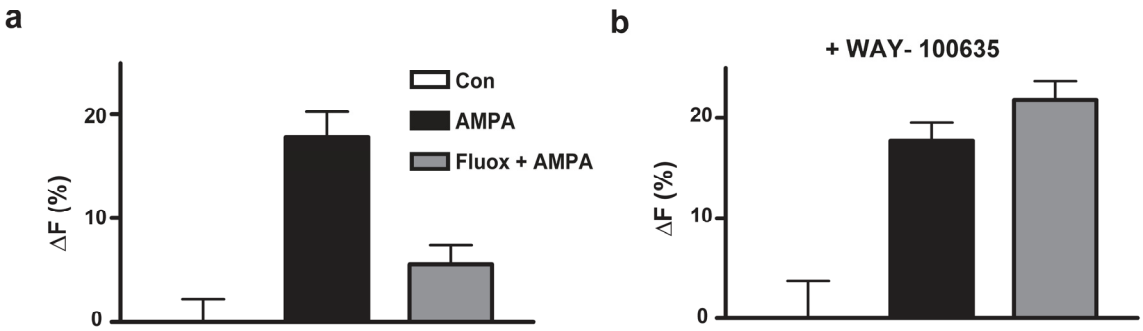

Figure 17. Autoreceptors inhibit somatic release in the presence of an antidepressant.

(a) Slices loaded with dHT were stimulated for 1 min with control solution (□), AMPA (■), or AMPA in the presence of 10 μM Fluoxetine (▭), a SERT inhibitor that is used to treat depression. Somatic ΔF (%) was quantified ($n \geq 16$). **(b)** Same as **(a)** except that $5HT_{1A}$ autoreceptors were inhibited with WAY 100635. The autoreceptor inhibitor was bath applied to slices 5 minutes before and during stimulation. (Con, n = 3; AMPA, n = 14; Fluox + AMPA, n = 11).

4.3.5 Detection of activity-dependent vesicular transport

In addition to assaying release, two-photon dHT imaging can detect vesicular transport during stimulation. Detection of vesicular transport can be measured by direct observation of extra-vesicular monoamine. Because release is vesicular, extra-vesicular monoamine is not affected directly by exocytosis. However, upon activation of vesicular transporters, extra-vesicular monoamine would be packaged into vesicles and thus decrease in concentration. Therefore,

vesicular transport should be revealed by measuring depletion of monoamine in a region free of vesicles. The nuclear envelope excludes vesicles, but is freely permeable to molecules <5 kDa (Gerace and Burke, 1988). Hence, although the transmitter content of the nucleus has not been measured previously, we reasoned that vesicular transport, if induced, should be revealed by depletion of extra-vesicular monoamine measured in the nucleus.

Thus, dHT fluorescence was measured in the nucleus. Specifically, nuclei in dHT-loaded slices were marked with the nuclear stain Hoechst 33342 (Figure 18a). Then optical sections through the equatorial plane of each nucleus were identified and used to outline the nucleus in dHT images (Figure 18b, c). Alternatively, the dHT signal at the center of the cell body, which always fell within the nucleus (Figure 18a), was quantified in the absence of Hoechst stain. Both assays showed that dHT was present in the nucleus (Figure 18b, c), suggesting that neurotransmitters move freely throughout the cell body.

To test whether extra-vesicular monoamine in the nucleus is depleted upon stimulation, nuclear dHT fluorescence was quantified before and after AMPA application for 1 minute. Both assays of extra-vesicular monoamine revealed comparable AMPA-induced depletion (Figure 18d, $AMPA_H$ and $AMPA_C$). To confirm that this depletion reflects packaging of extra-vesicular monoamine, VMAT was acutely inhibited. Specifically, reserpine was applied to dHT-loaded slices for five minutes. This brief reserpine treatment alone did not evoke any change in dHT fluorescence, showing that vesicular content was not affected. Then AMPA was applied in the continued presence of reserpine to ensure that VMAT was inhibited during the release response. The presence of reserpine completely eliminated the AMPA-induced depletion of extra-vesicular monoamine (Figure 18d, $AMPA_C$ + reserpine). Therefore, activity induces vesicular transport, which depletes extra-vesicular monoamine from the nucleus.

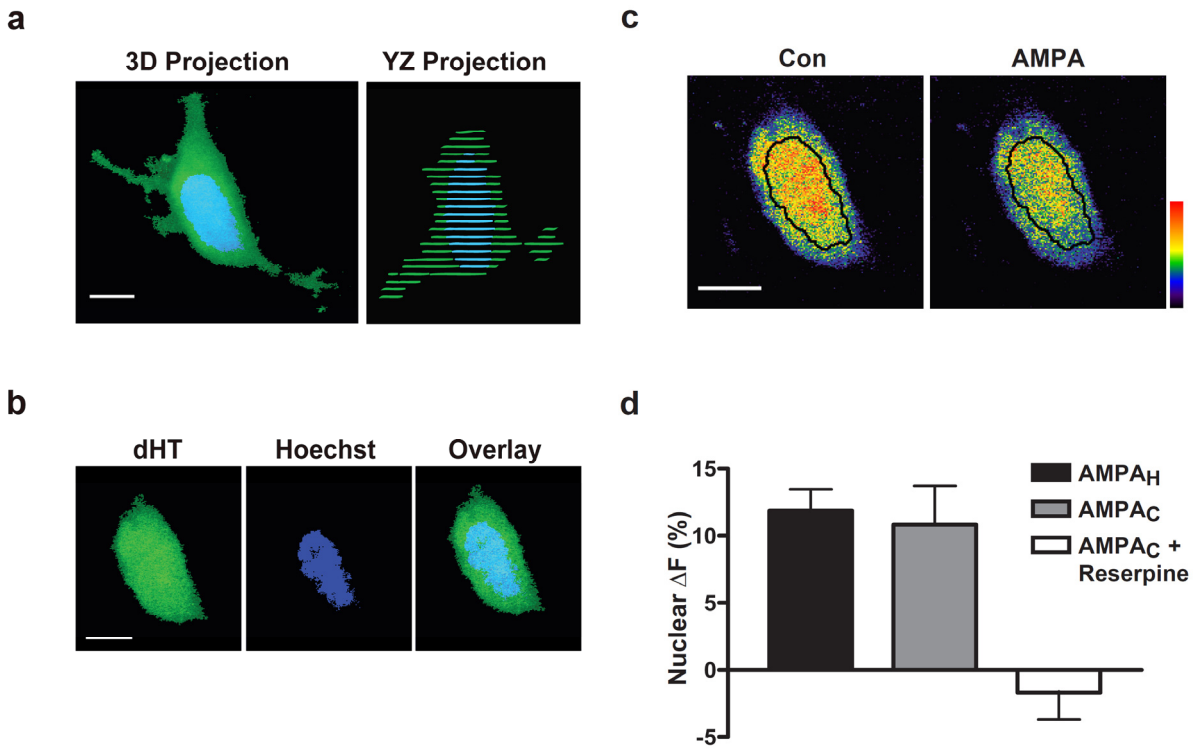

Figure 18. VMAT-mediated depletion of extra-vesicular dHT from the nucleus.

(a) 3D projection of a stack of optical sections through a dHT-loaded neuron stained with Hoechst 33342 to mark the nucleus (dHT, green; nucleus, blue). **(b)** Optical section through the center of the nucleus shows dHT (green) throughout the Hoechst-labeled nucleus (blue). **(c)** Pseudo-colored dHT equatorial section before (Con) and after AMPA stimulation. The nucleus as marked by Hoechst stain is outlined. **(d)** Quantification of the AMPA-induced decrease in nuclear fluorescence measured within the Hoechst defined nuclear border ($AMPA_H$; n=6) or the center of the neuron ($AMPA_C$; n=7). Acute application of reserpine just before and during stimulation blocked the AMPA-induced dHT depletion in the nucleus ($AMPA_C$ + reserpine; n=7). Scale bars = 10µm.

4.3.6 Quantification of VMAT-mediated packaging and release during stimulation

In order to determine the contributions of activity dependent vesicular transport and release to the somatic AMPA response, the vesicular dHT pool was quantified before and after stimulation. Quantification took advantage of the broad pH sensitivity of dHT, the ability to collapse the vesicular pH gradient with ammonium chloride and reserpine inhibition of VMAT. Application of 50 mM ammonium chloride (pH 7.4) for 30 s maximally increased somatic dHT fluorescence of resting neurons (Figure 19a). However, because ammonium shifted the pH of both vesicles (pH 5.5 → pH 7.4) and cytoplasm (pH 7.1→ pH 7.4), it was necessary to determine the portion of the fluorescence increase derived from the vesicular pool. Therefore, slices were incubated with reserpine during the initial dHT loading to prevent vesicular packaging of dHT. Application of ammonium to these slices led to a smaller fractional increase in fluorescence that represents the pH effect on extra-vesicular dHT. By subtracting this extra-vesicular fractional change from the total fractional change seen without reserpine, the effect of collapsing the pH gradient in VMAT-containing vesicles was measured (Figure 19b). The observed 13% increase in vesicular fluorescence coupled with the pH sensitivity of dHT (Figure 13b and 15b) indicates that 26% of total somatic content was packaged into secretory vesicles at rest. Ammonium experiments were then repeated on slices after AMPA treatment to determine vesicular content after stimulation. The 6% change in vesicular fluorescence observed with collapse of the pH gradient (Figure 18c) indicates that 12% of somatic content remaining after stimulation was packaged into vesicles.

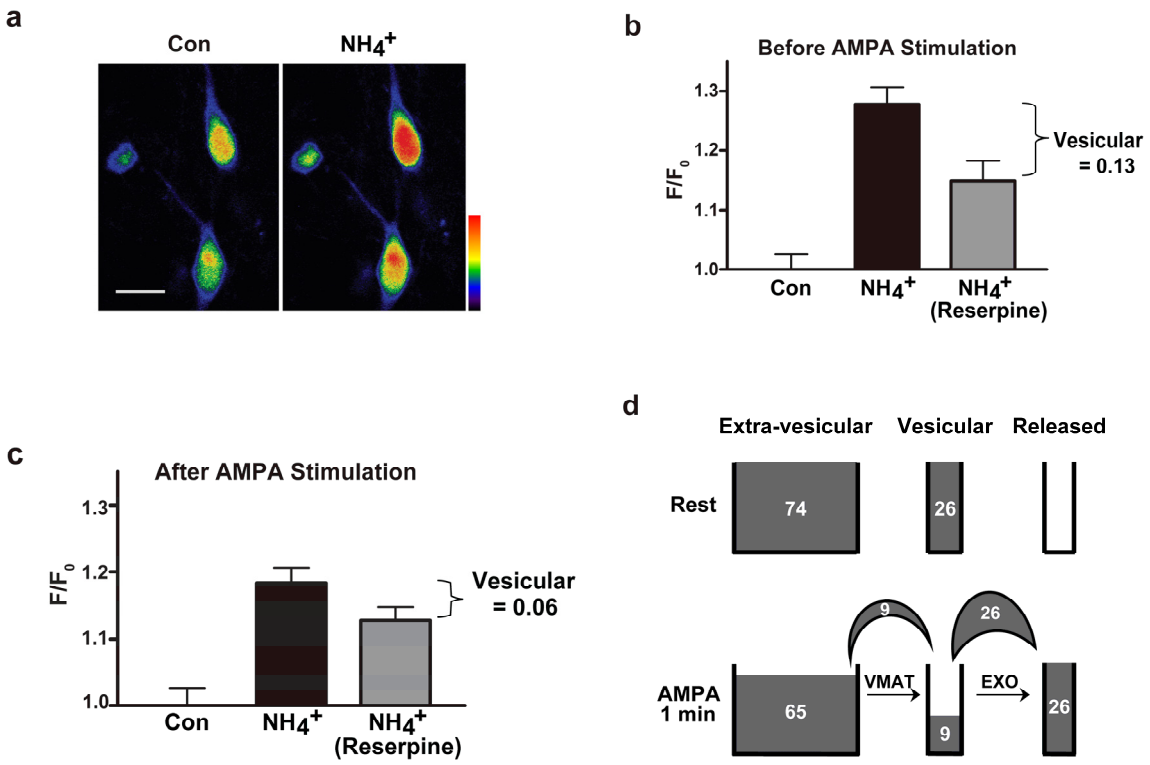

Figure 19. Vesicular content before and after stimulated release.

(a) Pseudo-colored summed dHT fluorescence before (Con) and after 30 s application of 50 mM ammonium chloride (NH_4^+). Scale bar = 20 µM. **(b)** Quantification of total (■) and extra-vesicular (▭) fractional fluorescence changes due to NH_4^+ application. The extra-vesicular change was measured in slices loaded in the presence of reserpine, which inhibits VMAT. The difference, indicated with a bracket, represents the NH_4^+-induced fractional fluorescence change in vesicular dHT ($n \geq 21$). **(c)** The experiment in **(b)** was repeated except that NH_4^+ was applied to slices after 1 min of AMPA stimulation ($n \geq 13$). **(d)** Summary of monoamine dynamics deduced from Fig. 18 and Fig 19. Quantification of vesicular content at rest (Fig 19b) allows deduction of extra-vesicular content at rest. Depletion of extra-vesicular content during stimulation (Fig. 18d) allows deduction of extra-vesicular content after AMPA (Eq. 1). Vesicular content after AMPA (Fig 19c) can be calculated in terms of original content (Eq. 2). Together this allows for the quantification of vesicular packaging and release during 1 min AMPA stimulation.

The above quantification of the vesicular pool at rest and after stimulation, together with the independently measured AMPA-induced change in the extra-vesicular pool (Figure 18d) can be used to calculate vesicular packaging and release during stimulation which is summarized in Figure 19d. Prior to stimulation, 26% of somatic dHT is packaged into vesicles (Figure 19b), which implies that the extra-vesicular pool contains 74% of somatic content. Stimulation depleted the extra-vesicular pool by 12% (Figure 18d), which corresponds to packaging of 9% of total somatic content (i.e., 0.09 = (0.74)*(0.12)). That leaves 65% of the initial total content in the extra-vesicular pool after stimulation (i.e., 74 - 9, also see Eq. 1). This last parameter coupled with the ammonia response after stimulation (Figure 19c) can be used to calculate that 9% of the total initial content remains in vesicles after stimulation (Eq. 2). Strikingly, the content that remains in vesicles after stimulation equals the amount that was packaged during stimulation (Fig. 19d, 9%).

These results lead to the conclusion that 26% of total content was released (Figure 19d). First, this amount equals the AMPA-induced decreases in the extra-vesicular and vesicular pools ([74-65] + [26-9]). Second, because the monoamine transported into vesicles during stimulation equals vesicular content after stimulation, an amount equivalent to the initial vesicular pool (26%) must have been released. Finally and most importantly, a prediction of the AMPA-induced change in fluorescence based on measurements of extra-vesicular content (Figure 18- nuclear depletion experiments) and vesicular content (Figure 19- pH collapse experiments) agrees with the independently measured somatic, AMPA-evoked fluorescence response (Figure 16). Specifically, taking into consideration the pH-dependent halving of dHT fluorescence in vesicles (Figure 13b, Figure 15b), the AMPA-induced dynamics summarized in Figure 19d predict a total ΔF of 20% (Eq. 3). This prediction is within the error of the measured AMPA-

induced somatic fluorescence response (18 ± 2.4%, Figure 16c), showing that results from diverse experimental designs all confirm the same conclusion (Figure 19d). Thus, the contributions of packaging and release to the somatic AMPA response have been quantified.

4.3.7 Efficient release of monoamine packaged during stimulation

The above results are compatible with a simple model in which all somatic vesicles undergo exocytosis once to completely release their preloaded content and then are refilled by VMAT (Figure 20ai). According to this hypothesis, VMAT activity during the 1 minute stimulation partially replenishes emptied vesicular stores, but does not contribute to release. However, an alternative hypothesis is that AMPA evokes release of only a portion of preloaded vesicular content. In this case, the rest of the response derives from efficient release of monoamine packaged by VMAT during stimulation (Figure 20aii). Preloaded vesicular content held in reserve would then account for the vesicular content detected after AMPA.

The above alternatives diverge with respect to the effect of acute inhibition of VMAT on release evoked by AMPA. According to the first hypothesis, blocking VMAT acutely during stimulation would not affect release because all release derives from monoamine that was packaged prior to AMPA application (Figure 20ai). In contrast, the second hypothesis predicts that acute inhibition of VMAT during stimulation would reduce release because monoamine packaged in the presence of AMPA contributes to release (Figure 20aii). In fact, AMPA-induced fluorescence changes during acute inhibition of VMAT can be predicted for each hypothesis based on the results presented thus far (Eq. 4). Specifically, if release is supported exclusively by preloaded vesicles (Figure 20ai), then AMPA stimulation after acute inhibition of VMAT

should evoke a 15% decrease in fluorescence (Figure 20b, upper dashed line i). On the other hand, if all transmitter packaged during stimulation is released (Figure 20aii), then acute inhibition of VMAT would reduce release to the fraction supported by preloaded vesicles. This would correspond to a 9.8% decrease in fluorescence (Figure 20b, lower dashed line ii). Finally, an intermediate balance between these two hypotheses would yield an intermediate result.

To inhibit VMAT during stimulation, reserpine was applied acutely (i.e., just before and during AMPA application) as described in previous experiments (Figure 18). AMPA stimulation after acute reserpine application evoked a 9.0 ± 2.0 % decrease in somatic fluorescence (Figure 20b). This result agrees with the second hypothesis (Figure 20aii): while some monoamine transported into somatic vesicles at rest is held in reserve, virtually all monoamine transported by VMAT during AMPA stimulation is released. Hence, activity-dependent vesicular transport is efficiently coupled to somatic release.

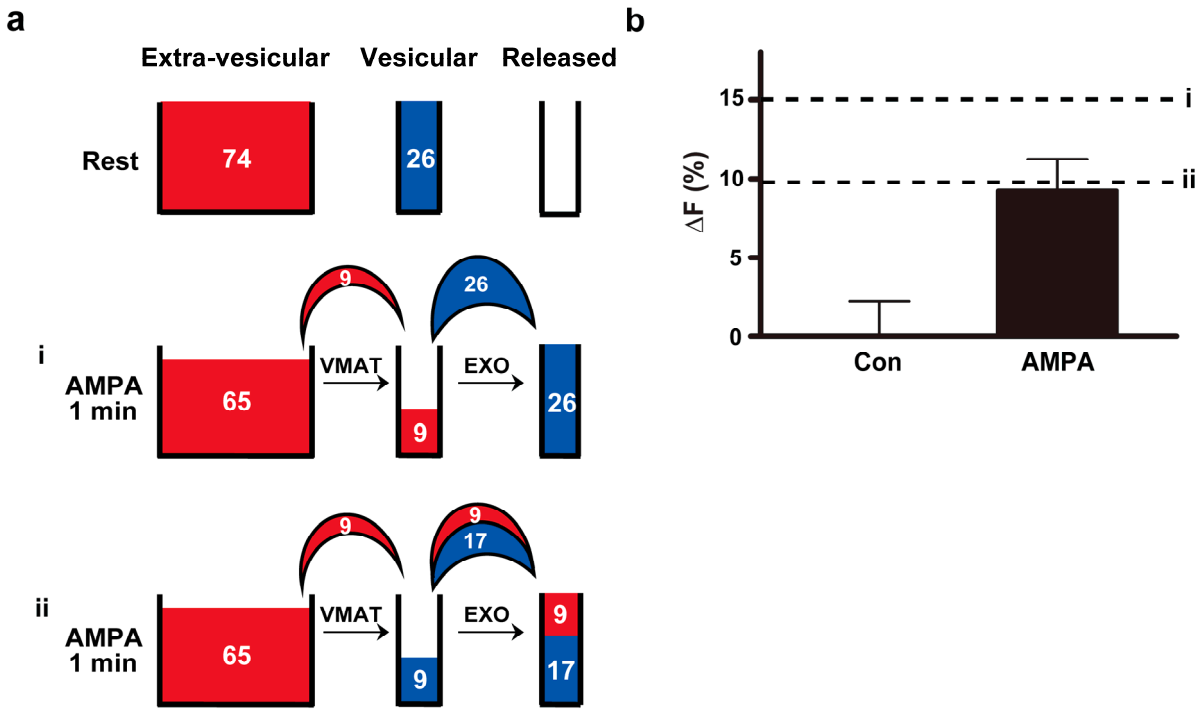

Figure 20. Activity-dependent contribution of vesicular transport to somatic release.

(a) Models of neurotransmitter packaging and release. Transmitter packaged in vesicles at rest (i.e. prepackaged) is indicated in blue. Extra-vesicular transmitter before stimulation is indicated in red. In model i, all prepackaged transmitter is released. Extra-vesicular transmitter is then packaged into vesicles. In model ii, release is supported by a portion of the prepackaged transmitter and all transmitter packaged during stimulation. Note that the activity-dependent contribution of VMAT to release is different in the two models: model ii relies on VMAT during the stimulus to contribute to released transmitter, while released transmitter in model i is independent of VMAT function during AMPA stimulation. (b) Quantification of somatic ΔF (%) in response to 1 min of control solution (Con) or AMPA ($n \geq 14$) in slices acutely exposed to reserpine during stimulation. Dashed lines show predicted AMPA responses for the models described in (a) (Eq. 4).

4.4 DISCUSSION

Here an approach for measuring vesicular transport and release in living DR serotonin neurons was developed to make fundamental insights into the activity-dependent function of vesicular neurotransmitter transporters. Although the use of a transmitter analog has limitations in assessing quantitative measurements, the ability to monitor VMAT activity during stimulation for the first time has allowed for several qualitative conclusions. First, while only a fraction of monoamine packaged at rest is released, release of monoamine packaged during activity is complete. This implies that basal VMAT activity does not discriminate between releasable and reserve pools. In contrast, during stimulation VMAT is active specifically in vesicles that rapidly undergo exocytosis. The coupling of activity-dependent vesicular transport and exocytosis is optimal for supporting sustained somatic release. Furthermore, this coupling provides an explanation for the classic observation that newly synthesized (and hence newly packaged) transmitter is released preferentially (Collier, 1969). Finally, these results reveal that both basal and activity-dependent vesicular transport contribute to somatic release by serotonin neurons.

Second, the nuclear compartment is relevant for vesicular transport in the serotonin neuron cell body. Previously, the role of the nucleus had not been considered because nuclear transmitter content had never been assayed. The permeability of the nuclear envelope, however, implies that endogenous transmitters in intact cells equilibrate between the cytoplasm and the nucleus. The nucleus occupies a large volume in the DR serotonin neuron cell body (Figure 18a) and does not contain monoamine oxidase, the enzyme responsible for intracellular catabolism of monoamine transmitters. Thus, the nucleus can serve as a large depot of diffusible transmitter

for somatic vesicle loading. The participation of the nucleus distinguishes monoamine dynamics at the serotonin neuron cell body from terminals.

The development of an assay that can measure vesicular transport in the context of neurotransmission will allow for future studies examining the regulation of quantal size. Emerging evidence suggests that regulation of vesicular packaging may define properties of neurotransmission during activity-dependent plasticity, exposure to drugs and during neuropathology (Edwards, 2007). However, the physiologic relevance and mechanisms of these changes had been difficult to assess due to the lack of a suitable live cell system. The regulation of VMAT can now be directly assayed in brain slice. Moreover, the relevance of VMAT regulation to neurotransmitter release can be studied.

Other aspects of serotonin neuron function are also amenable to study with two-photon dHT imaging. For example, antidepressant drug-dependent autoreceptor-mediated inhibition, which is thought to contribute to the delay of therapeutic efficacy of SSRIs (Blier et al., 1998), controls release from the cell body (Figure 17). Likewise, real-time SERT function in the brain slice can be assayed. Finally, two-photon dHT imaging is feasible with serotonin neuron dendrites and presynaptic terminals in brain slices (unpublished results). The optical detection of intracellular monoamine dynamics will complement other approaches to comprehensively examine neurotransmission by central serotonin neurons.

4.5 METHODS

4.5.1 PC12 cell experiments

PC12 cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37° with 5% CO_2. For imaging, cells were seeded on poly-D-lysine coated coverslips (#1.5, VWR) and transfected via Tfx50 (Promega) with HA-SERT and/ or eGFP. When indicated, PC12 cells were differentiated by application of 50 ng/ ml 2.5 S NGF for 36 hours. Five days after transfection, to allow for expression and trafficking of SERT, cells were incubated with 20 μM dHT (5,7-dihydroxytryptamine creatine sulfate, Regis Technologies), 568 μM ascorbate and 100 μM pargyline in culture medium for 1 hour or overnight. Overnight loading was followed by a 1 hour wash in culture medium at 37°C to allow cytoplasmic dHT to clear. Cells stably expressing ANF-emerald GFP(Han et al., 1999b) were transfected with SERT, differentiated, and loaded with dHT as described above. When cells were loaded in the presence of fluoxetine (10 μM) or reserpine (100 nM), drug was added along with dHT. For imaging, cells were bathed in normal saline (in mM, 5.4 KCl, 140 NaCl, 2 $CaCl_2$, 0.8 $MgCl_2$, 10mM Na-HEPES, 10 mM glucose; pH 7.4) and excited by epifluorescence or two-photon microscopy as described. For pH collapse experiments, monensin (1 μM) was bath applied during imaging. PC12 cells were stimulated by exchange of normal saline for high K^+ saline (in mM: 100 KCl, 45 NaCl, 5 $BaCl_2$, 0.8 $MgCl_2$, 10 Na-HEPES, 10 glucose; pH 7.4).

4.5.2 Slice Experiments

All experiments were conducted in accordance with protocols approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Male Sprague Dawley rats p14-p21 (Hilltop Labs) were anesthetized with isoflurane and decapitated. Brains were removed and bathed in 95% O_2 and 5% CO_2-saturated, ice-cold, sucrose-based artificial cerebral spinal fluid (s-aCSF; in mM: 87 NaCl, 75 sucrose, 2.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 7.0 $MgSO_4$, 25 glucose, 0.15 ascorbic acid, 1 kynurenic acid, pH 7.4). 250 μm coronal brain slices containing the DR were cut with a vibratome (The Vibratome Company) and incubated in 20 μM dHT, 568 μM ascorbate, and 100 μM pargyline in s-aCSF for 3 hours at 37°C. Slices were washed once in normal aCSF (in mM, 124 NaCl, 4 KCl, 25.7 $NaHCO_3$, 1.25 NaH_2PO_4, 2.45 $CaCl_2$, 1.2 $MgSO_4$, 11 glucose, 0.15 ascorbic acid, pH 7.4) before imaging. For slices loaded in the presence of fluoxetine (10 μM) or reserpine (50 nM), drugs were applied 10 minutes before addition of dHT. Stimulation of slices was induced by a bath exchange with aCSF supplemented with 10 μM AMPA. For stimulation with AMPA in zero calcium, $CaCl_2$ in normal aCSF was replaced with $MgSO_4$ and 1 mM EGTA was added. For autoreceptor studies, fluoxetine (10 μM) was added 5 minutes before stimulation with AMPA. The autoreceptor inhibitor WAY 100635 (2 μM) was added 5 minutes before fluoxetine application. When indicated, nuclei were stained by a 30-45 minute incubation of loaded slices with 1.6 mM Hoechst 33342 at the end of the experiment (i.e. after AMPA stimulation). For experiments requiring acute inhibition of VMAT, reserpine (50 nM) was added to slices 5 minutes before stimulation with AMPA. Finally, for pH collapse experiments, 50 mM NH_4Cl replaced 50 mM of NaCl in normal aCSF. NH_4^+ aCSF was applied to slices at rest or after AMPA stimulation as indicated.

4.5.3 Optical Setups

Widefield epifluorescence experiments were done on an Olympus IX71 inverted microscope equipped with a 340UV 40x 1.35 numerical aperture (NA) oil-immersion objective and a xenon arc lamp. DHT epifluorescence used a 360/40 excitation filter and 420 nm long-pass emission filter, which was collected by a cooled CCD camera (Hamamatsu Orca ER). Two-photon imaging experiments were done on an Olympus Fluoview FV1000 upright confocal scanning microscope. 725 nm excitation illumination from a Coherent chameleon ultra titanium sapphire laser was attenuated with an acoustical optical modulator and expanded with a motorized telescope (LSMtech) before being focused by a 60x, 1.1 NA water-immersion objective. Emission (400 - 480 nm) was quantified with a non-descanned detector (LSMtech), which contains a cooled Hamamatsu photomultiplier tube. Stacks of consecutive, 2.5 μm spaced images were taken through loaded neurons of the DR. All optical measurements were performed at room temperature.

4.5.4 Image Analysis

Analysis was done through Image J (NIH). Images were contrast enhanced or pseudo-colored to aid figure presentation without altering the primary data. When necessary, series of images were aligned with image j plug-in Stack Reg or stacks aligned with image j plugin Align 3TP. Mean fluorescence intensity was measured in regions of interest (ROIs) in single images or summed z-projections of image stacks and subtracted by background values (i.e. unloaded cells or tissue background fluorescence). Percent change in fluorescence (ΔF (%) = $(1 - (F/F_0)) * 100$) was normalized to control experiments to allow for comparisons across experimental manipulations.

'n' represents the number of cells from which data was collected. All data was compiled from at least three independent experiments. Error bars represent standard error of the mean.

4.5.5 Arithmetic Analysis

'v' is defined as the fraction of dHT in vesicles and 'c' as the fraction of extra-vesicular dHT. The subscript '$_0$' defines the variable in resting cells (i.e. before experimental manipulation). In all cases 'c' and 'v' are expressed in terms of fraction of total content at rest. Hence, $v_0 + c_0 = 1$. The fractional fluorescence of extra-vesicular dHT is denoted as 'Fc' and equals the fraction in extra-vesicular content 'c'. The fractional fluorescence of vesicular dHT is denoted 'Fv' and equals '½v' due to the pH-dependent halving of dHT fluorescence in acidic vesicles (Figure 13b, Figure 15c). Hence, the somatic fluorescence can be written in terms of the fractional dHT content in the vesicular and extra-vesicular pools ($F_0 = Fc_0 + Fv_0 = c_0 + (1/2)v_0$).

Equation 1: Figure 18 demonstrates a 12% change in the extra-vesicular fraction (ΔFc (%)) during AMPA stimulation. In order to calculate the extra-vesicular fraction after stimulation (c_{AMPA}) the following calculation was performed.

$$\Delta Fc\ (\%) = (1 - (Fc_{AMPA}/Fc_0)) * 100;\ c_0 = 0.74 \text{ (Figure 7b)}$$

$$12\% = (1 - (c_{AMPA} / 0.74)) * 100$$

$$c_{AMPA} = 0.65$$

Equation 2: Figure 19c demonstrates that 12% (Eq.1) of the somatic content after stimulation is in vesicles. In order to express the vesicular content after stimulation in terms of fraction of total content at rest, the following calculation is performed.

$$v_{AMPA} = 0.12\ (c_{AMPA} + v_{AMPA});\ c_{AMPA} = 0.65 \text{ (Eq. 1, Figure 6)}$$

$$v_{AMPA} = 0.12\ (0.65 + v_{AMPA})$$

$v_{AMPA} = 0.09$

Equation 3: A prediction of the AMPA-induced change in fluorescence can be made from measurements of vesicular and extra-vesicular content before and after stimulation (derived from Figures 18 and 19). This prediction can be compared to actual AMPA- induced fluorescence changes measured in Figure 16.

$$\Delta F_{pred}\,(\%) = (1 - (F_{AMPA}/ F_0)) *100$$

$$= (1 - (Fc_{AMPA} + Fv_{AMPA})/ (Fc_0 + Fv_0)) *100$$

$$= (1 - (c_{AMPA} + \tfrac{1}{2}v_{AMPA})/ (c_0 + \tfrac{1}{2}v_0)) *100$$

$$= (1 - (0.65 + \tfrac{1}{2}(0.09))/ (0.74 + \tfrac{1}{2}(0.26))) *100$$

$$= 20\ \%$$

Equation 4: Predictions of the change in fluorescence while acutely inhibiting VMAT during stimulation can be made for each model. Without AMPA-induced packaging of extra-vesicular dHT, the extra-vesicular pool is not depleted. Hence, $c_{AMPA} = c_0 = 0.74$.

$$\Delta F_{pred.}\,(\%) = (1 - (F_{AMPA}/F_0)) *100$$

$$= (1 - (Fc_{AMPA} + Fv_{AMPA})/ (Fc_0 + Fv_0)) *100$$

$$= (1 - (c_0 + \tfrac{1}{2}v_{AMPA})/ (c_0 + \tfrac{1}{2}v_0))*100$$

$$= (1 - (0.74 + \tfrac{1}{2}v_{AMPA})/ (0.74 + \tfrac{1}{2}v_0))*100$$

In model i all preloaded vesicular transmitter is released and then vesicles are refilled by VMAT. With acute VMAT inhibition, vesicles cannot be refilled. Therefore, no transmitter remains in vesicles after stimulation ($v_{AMPA}= 0.0$).

$$\Delta F_{pred.i}\,(\%) = (1 - (0.74 + \tfrac{1}{2}v_{AMPA})/ (0.74 + \tfrac{1}{2}v_0))*100$$

$$= (1 - (0.74 + \tfrac{1}{2}(0.0)/ (0.74 + \tfrac{1}{2}(0.26))) *100$$

$$= 15\ \%$$

In model ii only a portion of preloaded transmitter is released, while the rest is held in reserve and accounts for the vesicular content after AMPA stimulation. Because VMAT activity during the stimulus does not contribute to the amount remaining in vesicles after stimulation in this model, acute reserpine does not alter v_{AMPA}. Hence, $v_{AMPA} = 0.09$.

$$\Delta F_{pred.ii}\ (\%) = (1 - (0.74 + \tfrac{1}{2}v_{AMPA})/\ (0.74 + \tfrac{1}{2}v_0))*100$$

$$= (1 - (0.74 + \tfrac{1}{2}(0.09)/\ (0.74 + \tfrac{1}{2}(0.26))) *100$$

$$= 9.8\ \%$$

5.0 DISCUSSION

5.1 SUMMARY AND SIGNIFICANCE OF FINDINGS

Neurotransmission must be highly regulated while maintaining efficiency in order to mediate the complex and varied tasks demanded of it. Homeostatic and plastic changes must continually refine synaptic efficacy to respond to changing conditions. However, mechanisms that mediate this 'fine tuning' of transmission are not well understood. Classic interpretations of neurotransmission viewed neurotransmitter release as a fixed, stereotypic response to input, and thus limited mechanisms of plasticity primarily to changes in receptor responses. However modern understanding has now clearly defined an important role for the regulation of neurotransmitter release as well.

Release of transmitter is mediated by a series of highly regulated events including the packaging of secretory vesicles and their exocytosis. The packaging of transmitter is determined by the vesicular neurotransmitter transporter (VNT) family, whose presence and function are essential for neurotransmission. During both the biogenesis and the activity-dependent recycling of secretory vesicles, VNTs undergo trafficking that can determine the quality, quantity, and location of packaged neurotransmitter. Thus, understanding the signals and mechanisms of VNT trafficking are essential to understanding the regulation of neurotransmission.

In chapter 2 and chapter 3 of this thesis, the synaptic vesicle specific targeting of VNTs are investigated. Using the SV specific trafficking of VAChT in the PC12 neuroendocrine cell line as a model system, a specific synaptic vesicle targeting motif was identified. A chimera between an unrelated, plasma membrane localized protein and the C-terminus of VAChT trafficked to SVLVs, indicating the sufficiency of the C-terminus for SV targeting from the plasma membrane. Next, deletion analysis revealed the requirement and sufficiency of a 10 amino acid region containing a classic dileucine motif for both internalization and SVLV targeting. The duality of the motif as both an internalization and SV trafficking signal suggested efficiency in SV trafficking, consistent with the rapid kinetics of SV recycling at the terminal (Cousin and Robinson, 1999). Further examination of this motif revealed specificity for SVLV targeting over other internalization motifs and suggested that specificity lies in the type and environment of the motif. Specifically, while some dileucine motifs were not able to direct targeting to SVLVs, the closely related motif in $VMAT_2$ suggested that this signal may be generalized as a SVTM across several synaptic vesicle proteins.

In Chapter 3, sorting nexin 5 was identified as a novel regulator of the SVLV specific trafficking of VAChT. The characterization of SNX5 as a brain enriched protein that associates with SVs and the ability of SNX5 to bind to the C-terminus of VAChT suggested a functional interaction. Disruption of SNX5 by overexpression of mutant protein or siRNA mediated knockdown led to the mistargeting of the SVLV targeted protein to LDCVs. This mislocalization suggested a potential regulatory mechanism for membrane trafficking between different types of secretory vesicles. Thus, SNX5 was identified as a novel regulatory protein of SV specific trafficking, and suggested a potential mechanism of regulation that may be relevant for the modulation of neurotransmission.

Regulation of VNT trafficking or function has been reported to be involved in activity dependent plasticity, mechanisms of drug action, and disorders of the nervous system. This relevance suggests that VNTs may be an important pharmacological target (Iversen, 2000). However, in order to understand the regulation of VNTs, and the subsequent consequences for neurotransmission *in-vivo*, an assay that can measure vesicular transport and release in live neurons was necessary. Therefore, in Chapter 4, a novel method was established in order to measure these parameters. The use of a pH-sensitive, fluorescent serotonin analog, 5,7-dihydroxytryptamine, visualized with two-photon microscopy, allowed measurements of both vesicular transport and release in dorsal raphe nucleus serotonin neurons in the brain slice. Somatic release was accompanied by VMAT activity, which redistributed monoamine from the extra-vesicular compartment, including the nucleus. A portion of monoamine packaged at rest was held in reserve, however, all monoamine packaged during stimulation was released efficiently. This novel assay measured resting and activity dependent contributions of vesicular transport to release in living neurons for the first time and established a means of studying VNT regulation and the resulting modulation of neurotransmitter release.

The work presented in this thesis provides important information on the mechanisms and regulation of the synaptic vesicle trafficking of vesicular transporters. Moreover, it establishes a novel assay of vesicular transport in living neurons that allows study of activity dependent VNT function. The recent shift in the understanding of VNTs as passive 'gatekeepers' for secretory vesicles to targets of active regulation that define properties of neurotransmission has come as numerous studies conclude that VNT regulation is behaviorally relevant. These studies have implicated VNTs in the psychotropic action as well as the toxicity of drugs of abuse, the pathology of neurologic disease as well as the efficacy of pharmacologic treatments, and finally,

behaviorally relevant, activity-dependent plasticity. The centrality of VNT regulation to brain function should not be surprising due to the essential role of VNTs in neurotransmission. Despite this, the development of pharmacological agents to specifically target VNTs has received only limited attention. This thesis, in addition to providing novel information on the trafficking and activity-dependent function of vesicular transporters, lays a foundation for comprehensive studies on the active role of VNTs in shaping the properties of neurotransmission. Greater understanding in this area will undoubtedly yield advances in the development of novel therapies targeting VNTs.

5.2 REGULATION OF VACHT TRAFFICKING

5.2.1 Multiplicity of Pathways

The coexistence and relative prevalence of various recycling pathways including 'kiss and run' and clathrin mediated endocytosis has been a controversial topic (Rizzoli and Jahn, 2007). However, it is this coexistence of multiple pathways that would allow for the highly advantageous balance between efficient and regulated SV recycling that is likely required for neurotransmission. Activity- dependent recycling through a 'kiss and run' mechanism allows for faithful recycling of SVs in their current composition. In this case, vesicle proteins do not require sorting, as vesicle identity is maintained, and recycling is mediated by the closing of the fusion pore. Thus, this pathway is highly efficient, allowing rapid and faithful regeneration of SVs for further release. However, this mechanism does not allow for regulation of protein levels on the secretory vesicle, a potential means of plasticity.

On the other hand, clathrin mediated internalization, while less efficient allows for regulation of the SV proteome. A direct uncoating of internalized clathrin-coated pits to form SVs still maintains fast kinetics of recycling while allowing for modulation of the stoichiometry of SV proteins. Vesicle recycling through an endosomal intermediate may have slower kinetics and require multiple trafficking steps, however it allows for the greatest level of regulation. In addition to regulation of protein stoichiometry, recycling through an endosomal intermediate may regulate the balance of proteins between secretory vesicle types. LDCV proteins and SV proteins are seen together in endosomal intermediates, suggesting a potential site of component intermixing (Partoens et al., 1998). Moreover, trafficking of SV proteins through an endosomal intermediate may allow for independent regulation of vesicle subpopulations. Optical evidence supports the idea that pools of vesicles (i.e. readily releasable and reserve) use unique recycling pathways and that the properties of secretory vesicles in different pools are unique. Vesicles that are recycled through an endosomal intermediate are thought to populate the reserve pool of vesicles (Richards et al., 2000). Thus an endosomal pathway of recycling may provide 'storage' of a pool of vesicles (i.e. the reserve pool) with unique characteristics. This pathway may therefore provide a means for 'bulk' regulation, in which larger amounts of proteins and lipid are internalized together before precise sorting. Recruitment of the reserve pool for release, through changes in phosphorylation, could then serve as an efficient mechanism not only of recruiting vesicles for release, but also altering properties of neurotransmission.

Thus, the presence of multiple recycling pathways including 'kiss and run' and clathrin mediated internalization has been controversial for synaptic vesicles in the CNS. However it is precisely the coexistance of these multiple pathways that would allow for control between rapid

regeneration of SVs in their current composition and regulation of the properties of SVs, providing a balance between efficiency and plasticity that neurotransmission demands.

The question is then raised as to how the trafficking of specific proteins, such as VAChT, are regulated through these multiple pathways. First, the signals that mediate the trafficking through these pathways must be identified. A single dileucine-containing motif in the C-terminus of VAChT was identified as sufficient for both its internalization and synaptic vesicle targeting. Although this motif was identified under resting conditions, it is likely that this motif also directs VAChT targeting during activity-dependent vesicle recycling. Trafficking through a 'kiss and run' mechanism likely does not require targeting sequences. As vesicle identity is maintained during 'kiss and run', proteins do not need to be sorted. Thus, the identified dileucine motif is likely involved in clathrin-mediated pathways of recycling. The motif is consistent with a classic E(XXX)LL dileucine motif identified in non-neuronal, clathrin dependent internalization (Bonifacino and Traub, 2003). Moreover, the reported adaptor protein AP-2 binding to this dileucine motif in VAChT is consistent with trafficking through a clathrin-mediated recycling pathway (Barbosa et al., 2002). The presence of a single motif that has dual properties for both internalization and SV targeting suggests efficiency of trafficking and the ability of this motif to mediate direct SV formation from the plasma membrane. However, this does not exclude a role for this motif in mediating trafficking through an endosomal intermediate. The targeting of VAChT through an endosomal intermediate has not been explored directly. However, it is likely that endosomal pathways may be particularly relevant for VNTs, which can be found on multiple vesicle types and in multiple vesicle pools. The regulation of VNTs between vesicle types can define the relative content of vesicle types. In fact our studies from Chapter 3 suggest that VAChT trafficking has the potential to be regulated

between vesicle-types. It is likely that VAChT trafficking through an endosomal intermediate may also be mediated through the identified SVTM. However, the inability to distinguish between internalization signals and SV targeting signals within this regions suggests that the identified dileucine motif must act both at the plasma membrane for internalization and at the endosome for budding of the SV. Consistent with this, endosomal budding of SVs is thought to be mediated by an AP-3 dependent mechanism which has been shown to interact with dileucine motifs of the E(XXX)LL form (Bonifacino and Traub, 2003). However, interaction between VAChT and AP-3 has not been identified in binding studies (Kim and Hersh, 2004). This interaction may be transient in nature or occur only under certain conditions (i.e. under high levels of stimulation), thus it is difficult to interpret this negative result. However, it is also possible that VAChT may interact with unique cellular machinery.

The trafficking of VAChT through multiple trafficking pathways provides a means of increasing its regulation. However the mechanisms that determine the balance of VAChT trafficking between these recycling pathways needs further investigation. The prevalence of 'kiss and run' or full fusion mechanisms of endocytosis are thought to be regulated at least in part by activity (de Lange et al., 2003). In many cases, low frequency of stimulation leads to maintenance of synaptic efficacy through 'kiss and run' endocytosis. Under more intense stimulation, clathrin-mediated internalization through direct or endosomal trafficking may be most prevalent. One could hypothesize that it is precisely under these situations of high frequency stimulation that greater regulation of synaptic transmission is needed and that a clathrin mediated recycling pathway would allow for that regulation. Various readouts of synaptic activity, such as calcium levels, could regulate recycling machinery to induce a shift in recycling pathways. In fact, calcium mediated regulation of fusion pore collapse has been

reported (Fulop and Smith, 2006; He et al., 2006). Activity dependence may also regulate trafficking between a direct and endosomal mediated clathrin pathway. Shifts between these pathways could potentially be mediated by saturation of recycling machinery or machinery compartmentalization. Recently, the trafficking of VGluT was studied through the use of a chimeric protein between VGluT-1 and a pH-sensitive GFP variant in primary neurons. The trafficking of VGluT was regulated between an AP-2 dependent and an AP-3 dependent mechanism based on the intensity of stimulation. Regulation between these two pathways was hypothesized to be mediated by the competitive recruitment of VGluT-1 into the AP-2 dependent pathway by endophilin, an adaptor protein involved in clathrin mediated endocytosis (Voglmaier et al., 2006). Trafficking of VGluT through the two pathways relied on a common dileucine-like motif. This suggests that VAChT may be regulated similarly through multiple pathways relying on a single primary dileucine motif. The prevalence of recycling pathways may be regulated by interaction with regulatory machinery. Identification of trafficking proteins, like SNX5, that may regulate the prevalence of VAChT trafficking through different recycling pathways is needed.

5.2.2 Multiplicity of Signals

Analysis of the specificity and generality of the dileucine motif in VAChT suggested that a similar motif in $VMAT_2$ was also able to direct trafficking to SVLVs in the absence of a LDCV targeting sequence. Furthermore, dileucine-like motifs have been identified in several other SV proteins such as synaptotagmin and synaptobrevin 2 (Blagoveshchenskaya et al., 1999; Grote et al., 1995). However there is significant variability in the amino acid environments of these dileucine-like motifs. Furthermore, unrelated SV targeting motifs have been identified in other

proteins. In certain SV proteins, synaptic vesicle trafficking signals do not appear to exist and their SV-specific location relies on their association with other SV proteins (Pennuto et al., 2003). This multiplicity of signals has made understanding the regulation of SV targeting difficult. Furthermore, the reconciliation of a large number of trafficking motifs with a limited number of common trafficking pathways has been difficult.

One possible explanation of the multiplicity of signals identified in the SV targeting of proteins is to allow for individual recruitment and regulation through common pathways. Two proteins may have signals that direct them through clathrin and AP-2 dependent internalization and SV targeting from the membrane, however, they may rely on unique protein interactions that distinguish the efficiency of their recruitment. The signals that mediate this trafficking may have some similarities such as a dileucine-like motif, however the environment of the motif or additional motifs may regulate the efficiency or recruitment into clathrin pits or association with other SV proteins that are co-regulated.

The presence of a unique and sufficient dileucine motif in VAChT allows it to be individually regulated during SV recycling. Modulation of VAChT recycling efficiency to alter VAChT protein levels on vesicles is likely regulated by additional factors that remain to be identified. One such factor could be the phosphorylation state of the dileucine motif of VAChT by PKC, which has been shown to alter the SV trafficking of VAChT (Cho et al., 2000). This may reduce the efficiency of internalization or alter interactions with adaptor proteins. Additionally, the presence of two tyrosine based motifs in the C-terminus of VAChT have been suggested to regulate the efficiency of VAChT internalization or SV targeting, although the mechanisms of this have not yet been determined (Kim and Hersh, 2004).

5.2.3 Potential mechanisms of SNX5 regulation

The identification of sorting nexin 5 as a regulator of the synaptic vesicle trafficking of VAChT confirmed the involvement of specific protein interactions in the regulation of VAChT trafficking. The mislocalization of the VAChT chimera to LDCVs upon the disruption of SNX5 function suggested that SNX5 regulates the trafficking of VAChT between secretory vesicle types. Although VAChT has been characterized as highly enriched in SVs, it has been identified to reside to a lesser extent on LDCVs (Agoston and Whittaker, 1989; Lundberg et al., 1981). Moreover the finding that disruption of the interaction between SNX5 and VAChT can shift trafficking between secretory granules defines an important potential mechanism of regulation that may be common to other VNTs such as VMAT, which is often found in multiple vesicle types in a single neuron. In fact, preliminary evidence suggests that SNX5 may bind $VMAT_2$ (unpublished data). The mechanism of SNX5 regulation remains to be identified, however potential models are explored below.

Regulation between secretory vesicle types may suggest a common pathway of SV and LDCV biogenesis. Classically, proteins destined for different vesicle types were thought to segregate at the level of the TGN into the regulated and constitutive secretory pathways. However, certain SV bound proteins may take an alternate route. These proteins may traffic into the regulated secretion pathway. This could be due to the presence of targeting motifs similar to LDCV bound proteins, association with other proteins bound for this pathway, or inefficient TGN sorting. During the maturation of LDCVs, proteins destined for other locations in the cell are removed in a more stringent sorting process by the budding off of transport vesicles. This sorting process is an AP-1, ARF1, and GGA dependent process (Dittie et al., 1996; Kakhlon et al., 2006).

Interestingly, VAChT has been shown to bind to AP-1 through its C terminus dileucine motif and thus has been suggested to traffic in this manner to SVs (Kim and Hersh, 2004). This alternative biogenesis pathway suggests a common initial pathway of VNTs destined for both types of secretory vesicles, which would allow direct regulation of trafficking between them. The data presented in Chapter 3 suggests that SNX5 may regulate this trafficking. In the model of biogenesis proposed above, SNX5 would promote the budding of VAChT from immature secretory granules, removing it from the regulated secretory pathway. Disruption of SNX5 function, or the interaction between SNX5 and VAChT, would therefore prevent the removal of VAChT from immature granules and lead to its accumulation in LDCVs.

This potential model of regulation is consistent with the known properties of SNX5. The role of the sorting nexin family in regulating the trafficking of membrane proteins has been well defined, although the mechanisms of this regulation are not clear (Carlton and Cullen, 2005). The presence of a curvature sensing/forming BAR domain in SNX5 suggests that is likely involved in trafficking of proteins via a budding mechanism. The PX domain of the sorting nexin family was originally characterized for its affinity for the phosphoinositide PI_3P lipids, localizing the sorting nexin proteins to membranes enriched in these lipids. However, recent evidence has suggested that unique amino acid sequences in various PX domains of the members of the sorting nexin proteins may mediate different phospohinositide specificity. Recently, SNX5 has been characterized in non-neuronal cells. Its PX domain has shown affinity for PI_4P, which is enriched in TGN derived membrane (Liu et al., 2006). Moreover, PI_4P recruits GGAs and AP-1 and thus SNX5 is well suited to be involved in membrane trafficking involving these characteristics. Thus, properties of SNX5 in non-neuronal cells suggests characteristics that are consistent with regulating trafficking from immature secretory granules in neurons.

SNX5 has also been recently characterized through genetic studies as a potential component of the mammalian retromer complex (Wassmer et al., 2007). Classically, the retromer complex is thought to mediate, endosomal/lysosomal to TGN trafficking, best described in the recycling of the cation independent mannose-6-phosphate receptor (CI-MPR) after delivery of its cargo to the lysosome (Bonifacino and Hurley, 2008). Consistent with this role, our lab has previously demonstrated that SNX5 inhibits the degradation of EGFR from late endosomes (Liu et al., 2006). A role for the retromer in regulating targeting between secretory vesicle types is not apparent, however the CI-MPR is known to traffic through immature secretory granules. This receptor is removed during LDCV maturation in an AP-1 dependent process as described above (Klumperman et al., 1998). Thus, SNX5 which may play a role in CI-MPR trafficking as a component of the retromer, may also play a role individually or as a larger protein complex in the removal of this receptor and other proteins from the regulated secretion pathway, including VAChT. The trafficking pathway of VAChT once removed from ISGs remains to be determined. Vesicles budding off of ISGs have been reported to traffic to various locations including the plasma membrane in a constitutive-like pathway, the TGN, or endosomes, all of which could be intermediates in the formation of SVs (Tooze and Stinchcombe, 1992).

This biosynthetic pathway is also consistent with the known regulation of VMAT trafficking between secretory vesicles. The C-terminus acidic patch in VMAT may disrupt interaction with regulatory machinery, such as SNX5, that mediates sorting away from the regulated secretory pathway. In this way, this motif would promote retention to the regulated secretion pathway and increase VMAT targeting to LDCVs. Interestingly, phosphorylation of residues in the acidic patch promotes a shift in $VMAT_2$ targeting to SVs (Waites et al., 2001).

Thus, the inclusion of $VMAT_2$ in vesicles budding off of ISGs may be regulated by phosphorylation.

Interestingly the finding that SNX5 associates with synaptic vesicles (Figure 11; (Takamori et al., 2006) suggests that SNX5 may have a more permanent interaction with VAChT than described above. Thus, SNX5 may regulate VAChT targeting at multiple steps in the SV trafficking pathway. As mentioned previously, the endosome is another potential site of regulation between secretory vesicles. Proteins from both LDCVs and SVs intermix in endosomal compartments after stimulation and must be sorted (Partoens et al., 1998). Interaction of VAChT with SNX5 may therefore regulate SV sorting in the endosome. Proteins that are not selectively sorted for SV retention at this stage may be trafficked to the lysosome for degradation or the TGN for further sorting into secretory pathways as described above. Interestingly, several studies have indicated an endosomal localization of SNX5 in non-neuronal cells, suggesting that it may play a role in trafficking steps at the early endosome (Merino-Trigo et al., 2004; Yoo et al., 2006). The identification of a regulatory protein that may act to direct SV specific trafficking of VAChT at several steps of a trafficking pathway is appealing as it is both efficient and provides for redundant levels of regulation. This may be particularly relevant for the VNT family of proteins whose location to secretory granules is essential for neurotransmission and whose trafficking properties define the vesicle type and amount of packaged neurotransmitter. It is interesting to note that interaction of SNX5 showed at least some specificity of regulating VNT traffic as trafficking of another SV protein, synaptophysin, was not regulated by SNX5 (Figure 12).

Further studies that examine the interaction of SNX5 with other SV proteins would be of great interest. In particular, confirmation of the functional interaction of $VMAT_2$ and SNX5

would help to define the mechanisms of SNX5 function. As mechanisms that mediate regulation of $VMAT_2$ targeting between vesicle types have been identified, such as changes in phosphorylation in the acidic patch, further study on the regulation of the interaction between SNX5 and $VMAT_2$ may provide more insight into physiologic regulation of VNT trafficking. Confirmation of VAChT trafficking through the ISGs as well as further study on the endosomal trafficking of VAChT will allow for greater understanding of the sites and mechanism of SNX5 regulation.

5.2.4 Future Studies of VNT trafficking

Further elucidation of mechanisms that regulate the trafficking of VAChT and other VNTs is necessary. Future studies will have to examine trafficking motifs and machinery not only under resting, steady-state conditions, but also during stimulation. Multiple stimulation conditions to examine the prevalence of one recycling pathway over another will be helpful to identify cytosolic machinery responsible for trafficking regulation. Moreover, it will be important to incorporate multiple identified motifs and regulation of VNT trafficking into a cohesive picture of SV recycling. Finally, although the primary focus has been on SV trafficking at the synapse, understanding the polarized targeting of VNTs, which may regulate the axonal/somatodendritic localization of the proteins will be important to examine. It is important to note, that experiments that study trafficking will need to examine the efficiency of trafficking rather than an end-point read out to understand trafficking regulation. Moreover, studies that are able to examine trafficking of multiple synaptic vesicle proteins, or the trafficking of SV proteins relative to cycling lipid, will increase our understanding of the relative regulation of SV proteins that define vesicle protein stoichiometry. This will require a combination of sensitive and

dynamic techniques including optical, biochemical and genetic methodology. A greater understanding of mechanisms that mediate the selective regulation of VNT trafficking will allow us to understand complex mechanisms of regulation used to define synaptic transmission such as the recent identification of diurnal trafficking regulation of VGluT (Darna et al., 2008) as well as other regulation that may act to acutely regulate properties of transmission.

5.3 PHYSIOLOGIC RELEVANCE OF VNT REGULATION

The measurement of VNT function in the context of neurotransmission in living neurons holds great significance for the field of VNTs. For the first time potential mechanisms of VNT regulation can be studied in intact neurons and the physiologic relevance of this regulation can be simultaneously measured. Moreover, the establishment of this assay allowed for direct measurements of the relative contribution of activity-dependent vesicular transport to release. The use of a false transmitter in this assay suggests the quantitative measurement of this contribution may not be indicative of native transmitter. As the handling properties (i.e. km and Vmax for VMAT or SERT) and/or the cytosolic concentration of the false transmitter are likely not precisely the same as that of the native transmitter, the quantitative measurements of vesicular transport and release may not be reflective of native responses (although its not obvious in what direction the use of DHT rather than native transmitter might affect these values). However, while the quantitative measurements of vesicular transport may not be precise, the qualitative conclusion, that transmitter packaged during stimulation is released efficiently holds true.

This conclusion underscores the importance of the VNT family of proteins as an efficient means of regulating properties of transmission. There are at least two possible interpretations of the transmitter dynamics that mediate this result. One is that upon stimulation $VMAT_2$ activity is upregulated specifically in a pool of vesicles that will undergo release, loading additional transmitter in this subset of vesicles before release. This subset of vesicles would likely comprise a readily releasable or 'primed' pool of vesicles. The association of this pool of vesicles with certain machinery or the conformation of certain proteins within these vesicles may serve to distinguish this subpopulation and allow for the unique regulation of VMAT within this group of vesicles. Interestingly, a recent study suggested that the vesicle pre-fusion protein Ca^{+2} dependent activator of protein secretion (CAPS) has been shown to increase vesicular monoamine transporter function, suggesting a potential mechanism of the unique regulation of vesicular transport in vesicles destined for release (Brunk et al., 2009). This potential regulation would be a very efficient mechanism of altering quantal size and is deserving of further study.

An alternative interpretation of the results presented is that upon stimulation a pool of vesicles undergoes exocytosis, refilling and subsequent exocytosis. Vesicles that are refilled undergo exocytosis such that virtually everything that is packaged during stimulation is released. This may suggest that vesicles are rapidly cycling compared to the one-minute time point of analysis. Estimates of vesicle reacidification, through studies using pH sensitive fluorescently tagged SV proteins demonstrated kinetics on the order of a few seconds, consistent with the potential for multiple cycles of exocytosis within one minute (Atluri and Ryan, 2006). Furthermore, vesicle cycling studies have suggested that recently recycled vesicles are released preferentially, thus defining a subset of cycling vesicles (Pyle et al., 2000). These observations are consistent with the observations that vesicles pools may recycle within themselves (Richards

et al., 2000). Thus, vesicles that are released from the readily releasable pool recycling back into a readily releasable pool. This mechanism, which implies trafficking of VNTs through the recycling of a subset of SVs, suggests that regulation of VNT trafficking has the potential to influence release acutely. Interestingly, recent work at CA-1 pyramidal terminals showed an increase in synaptic depression upon the blockade of vesicle acidification within milliseconds (Ertunc et al., 2007). The speed of synaptic depression induced depended on stimulation frequency, implying an activity-dependent regulation of vesicle trafficking through recycling pathways of different kinetics. This is consistent with the potential for regulation of VNT trafficking to acutely affect release. The dependence of activity dependent vesicular transport on vesicle cycling can be tested through inhibition of vesicle endocytosis to clarify our results.

An important characteristic of the studies done in Chapter 4 that has not been expressly discussed is that release and vesicular transport were measured at the somatodendritic compartment of Raphe neurons and not at terminals. The classic observation that neurons are unidirectional in nature has clearly been redefined as somatodendritic release of transmitter has been described for many classes of neurons. Mechanisms that mediate somatodendritic release are not as well understood and appear to vary by cell type. However, somatodendritic release in the Raphe nucleus has been shown to be vesicular and calcium dependent and thus may share many of the same mechanisms as release from terminals (de Kock et al., 2006). However, the role of vesicle cycling or vesicular transport at the somatodendritic compartment has not been explored. One major difference in vesicular release from terminals and somatodendritic compartments in the serotonin neurons of the Raphe is the absence of defined synaptic specializations in the latter. While clusters of small vesicles are often seen in somatodendritic compartments, they do not appear to be associated with presynaptic specialties. Thus, any

cycling of vesicles in the soma is occurring in the absence of the characterized active zones present at axon terminals. It is unclear whether the same complement of trafficking machinery proteins are present at release sites in somatic compartments. Further study examining the protein composition and morphology of release sites in the soma as well as optical studies of vesicle cycling in this compartment are needed. The efficient release of transmitter packaged during stimulation therefore suggests remarkable and perhaps unexpected efficiency of vesicular transport and release at the somatic compartment.

Whether mediated through selective activation of $VMAT_2$ in a subset of vesicles or the rapid cycling of vesicles through rounds of release, the results presented demonstrate that activity-dependent vesicle filling contributes to release on an acute time scale. This suggests that regulation of VMAT function, through trafficking or other means, would have an immediate impact on release. Thus, regulation of VNT function can induce acute changes in neurotransmission.

5.4 CONCLUDING REMARKS

While recent work has identified the potential of VNT regulation as a means of synaptic plasticity, the mechanisms of this regulation as well as the physiologic significance is not well understood. In this thesis, I have identified important properties of VNT trafficking and function. Specifically, I have identified a SVTM necessary and sufficient to mediate SVLV targeting of VAChT in the neuroendocrine PC12 cell line. Moreover, I have identified SNX5 as a novel regulator of this trafficking. Disruption of SNX5 function revealed a potential mechanism of regulation between secretory vesicle specific trafficking. Finally, an assay was

established to measure vesicular transport in live neurons. Measurements of vesicular transport and release revealed the efficient release of transmitter packaged during stimulation. This collection of work has revealed important properties of VNT trafficking and activity-dependent function and has laid the foundation for studying the regulation of VNT function as a means of modulating neurotransmitter release.

The future of the VNT field lies in understanding the mechanisms that regulate VNT targeting during stimulation, drug application or disease pathology. Studies that move beyond identification of steady-state trafficking properties and examine the efficiency of trafficking over time or during changing conditions, rather than end point readouts, will be most informative. The identification of mechanisms that regulate the efficiency of VNT trafficking allow for the potential of manipulating synaptic efficacy. The development of an assay for vesicular transport in live cells will be invaluable in future studies of VNT regulation. Not only will this assay allow for the exploration of mechanisms that regulate vesicular transport, but will also allow for the analysis of the physiologic relevance of this regulation in terms of neurotransmission. Additional assays that will allow for measurements of vesicular transport in other neurotransmitter systems and that provide spatial discrimination of vesicles will further enhance the progress of the field. The appreciation of VNT regulation as a means to modulating synaptic efficacy will hopefully lead to a greater focus of the VNT family of proteins as potential therapeutic targets.

APPENDIX

SUPPLEMENTAL INFORMATION: SORTING NEXIN 5 REGULATES THE SYNAPTIC VESICLE SPECIFIC TARGETING OF VESICULAR ACETYLCHOLINE TRANSPORTER

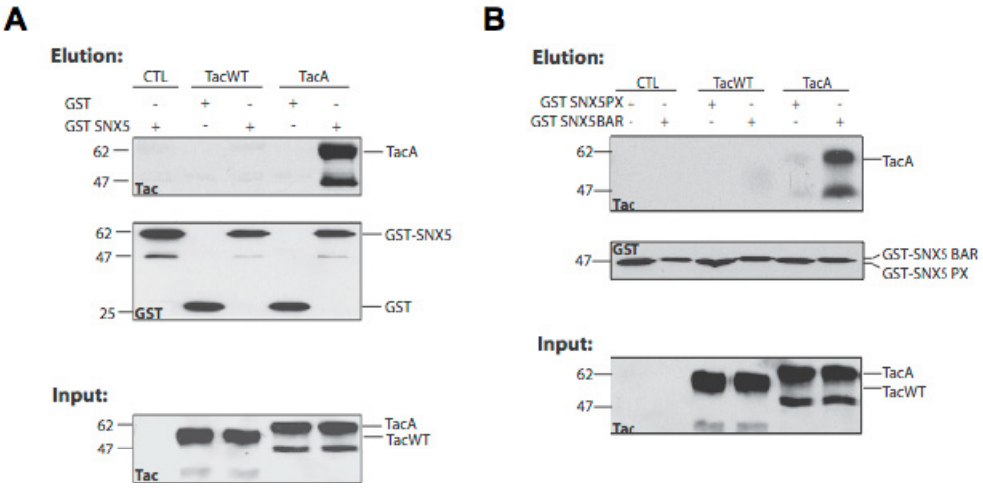

Figure S 1. Snx5 binds to VAChT C-terminus

A) HEK293 cell lysates, transiently transfected with Tac (TacWT) or the chimeric protein between Tac and the C – terminus of VAChT (TacA) were incubated with glutathione-sepharose immobilized GST or GST-SNX5 as indicated. Bound protein was eluted and detected by Western blot. GST-SNX5 pulled down TacA but not TacWT. B) Interaction is mediated by GST-SNX5BAR domain as indicated by its selective binding to TacA.

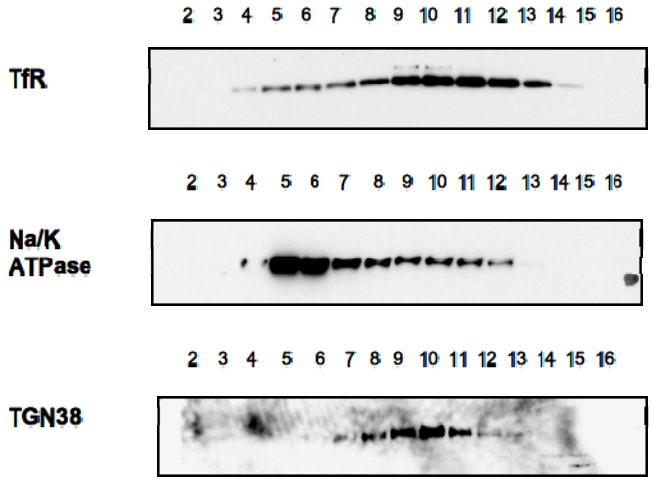

Figure S 2. Subcellular Markers in Gradient Fractionation

Markers of subcellular compartments from siRNA SNX5 sucrose gradient in Figure 2B migrate to unique fractions in sucrose velocity gradient. Transferrin Receptor (TfR) marks endosomal compartments. Na/K ATPase marks plasma membrane. TGN38 marks the trans golgi network.

METHODS

Chemicals and antibodies

Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. The following antibodies were used in immunofluorescent staining: biotin conjugated mouse anti-CD25 (Affinity BioReagents, Golden, CO), polyclonal synaptophysin and secretogranin II (SYSY, Goettingen, Germany), secondary Cy3 conjugated goat anti-mouse and Alexa 488 conjugated goat anti-rabbit (Jackson Immunoresearch Lab, West Grove, PA). The following antibodies were

used for Western blot: polyclonal IL-2R alpha (Santa Cruz Biotech., Santa Cruz, CA), monoclonal anti-IL2 Receptor (Covance, Princeton, NJ), polyclonal anti-synaptophysin and anti-secretogranin II (SYSY, Goettingen, Germany), secondary HRP conjugated goat anti-mouse and goat anti-rabbit (Pierce, Rockford, IL).

Plasmid construction and mutagenesis

Chimeric protein TacA was generated as previously described (citation) . Briefly, the C-terminus of VAChT (a.a. 465-530) was amplified by PCR with the introduction of Xba1 and Xho1 restriction sites flanking the region. PCR product was digested and subcloned into Tac/pcDNA 3.1 as described (Tan et al., 1998). Glutathione *S*-transferase (GST)-tagged SNX5 and SNX5PX, SNX5BAR plasmids were generated by cloning full length SNX5 (1-404), SNX5PX (1-179), or SNX5BAR (180-404) cDNA into pGEX-6p-1 vector (Amersham) in frame with the GST coding sequence. Similarly, HA-tagged SNX5 and its truncated mutants were made by inserting cDNAs into a modified pcDNA3.1 vector in frame with the HA coding sequence. All constructs were confirmed by sequencing.

Cell culture and transfection

All cells were maintained in 5% CO_2 at 37ºC in medium containing penicillin and streptomycin unless otherwise noted. PC12 cells were maintained in DMEM (Invitrogen) with 10% Equine serum (Hyclone, Logan, UT), 5% Cosmic Calf serum (Hyclone, Logan, UT), and 2 mM L-Glutamine (Invitrogen). Stable lines of wild type Tac, TacA, were generated as previously described (Colgan et al., 2007). Hek293 cells were grown in DMEM with 10% fetal bovine serum (Hyclone, Logan, UT). All cells were transfected using LipofectAMINE 2000

(Invitrogen, San Diego, CA) reagent or electroporation according to the manufacturer's instructions. PC12 cells were differentiated using 100 nM NGF for 24 hours. Transfected cells were incubated at 37ºC for 36 - 48 hours before harvest.

Immunofluorescence and confocal microscopy

Immunofluorescent staining was performed as previously described (Liu et al., 2006). In brief, cells seeded on glass coverslips were fixed with 4% paraformaldehyde in PBS, pH 7.4. After fixation, cells were permeabilized and blocked for 30 min. in blocking buffer (BB, 2% BSA, 1% fish skin gelatin, and 0.02% saponin in PBS). Cells were then incubated with primary antibody in BB for 1 hour at room temperature. Coverslips were washed and incubated with the appropriate Alexa- or Cy3- conjugated secondary antibody for 1 hour at room temperature. Confocal images were acquired with a Fluoview 500 laser scanning confocal imaging system (Olympus, Tokyo, Japan) configured with a fluorescence microscope fitted with Pan Apo 60× and 100× oil objectives (Olympus). Confocal images were collected sequentially at 1024×1024 resolution to minimize bleed through of fluorescence between channels.

Yeast-two-Hybrid

The MATCHMAKER LexA yeast two-hybrid system (BD Clontech) was used to identify the interacting proteins for VAChT. The C-terminus of VAChT was used as bait to screen a PC12 cDNA library as described in the user manual (BD Clontech). Plasmids for both cDNA library and bait were transformed sequentially into yeast strain EGY48 using the lithium acetate method and cultured on SD selection medium according to the manufacturer's manual (PT3040, BD Clontech). The yeast transformants were restreaked on SD/His^-/Trp^-/Leu^-/Ura^- induction medium

plates containing Galactose, Raffinose and X-gal. Only transformants displaying blue color within 3 days were considered as positive result of protein-protein interaction. Positive colonies were restreaked for single colonies and then the cDNA plasmids were retrieved and the contained cDNA inserts were PCR amplified for sequencing.

Immunoprecipitation

Expression and purification of GST fusion proteins were done as described previously (Liu et al, 2006). 40ug of GST-fusion proteins or control GST protein coupled to glutathione sepharose beads were incubated with transfected HEK293 cell lysate at 4°C overnight. Beads were then carefully washed for four times with washing buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl, 0.1% NP-40 and protease inhibitors). The bound proteins were eluted using SDS–PAGE sample buffer and were subjected to SDS–PAGE and immunoblotting with specific antibodies. Protein bands were visualized by the ECL detection system (PIERCE).

Sucrose Gradient Fractionation

PC12 cells stably expressing, or transiently transfected with plasmid constructs, were harvested in Buffer A (150 mM NaCl, 10 mM Hepes pH 7.4, 1 mM EGTA, 0.1mM MgCl2) with protease inhibitors. Cells were cracked by eight passes through a cell cracker (Clearance-0.02um). Post-nuclear supernatants (PNS) were then loaded onto prepared density gradients and spun in a Beckman SW41 rotor. For sucrose density fractionation, sucrose gradients were prepared using a gradient mixer to form continuous gradients with sucrose concentrations from 0.65 M to 1.55 M and spun in SW41 rotor at 30,000 rpm for 8 hours at 4°C. (Beckman Instruments, Palo Alto, CA). Aliquots were taken from each gradient for western analysis as described below.

Two-step gradient for LDCV purification

LDCVs purification is performed by sequential fractionation through two sucrose gradients (Stinchombe and Huttner, 1994). PC12 cells were loaded overnight with 2.5 μCi/ml [^{3}H] norepinephrine to label LDCVs. PNS was prepared as described above in the presence of protease inhibitors. PNS was layered onto a 0.3-1.2 M sucrose gradient and sedimented at 25,400 rpm in an SW41 rotor for 30 min at 4°C. Fractions were collected and radioactivity measured in an aliquot of each fraction by scintillation counting. Pooled fractions containing the bulk of the radioactivity were then loaded onto a 0.65-1.55 M sucrose gradient and sedimented to equilibrium at 30,000 rpm in an SW41 rotor for 6-12 hours at 4°C. Collected fractions were loaded onto gels for western blotting detection as described.

Western blot analysis

Proteins were detected in gradient fractions by immunoblotting as previously described (Chen et al., 2005). Equal amounts of gradient from each fraction were denatured in 3x SDS sample buffer (New England Biolab, Beverly, MA), and separated by electrophoresis through 10% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose (BA-85, Schleicher-Schuell Bioscience, Keene, NH) and TacA chimeric proteins, synaptophysin, secretogranin II, or other marker proteins were visualized by immunoblotting with appropriate antibodies in combination with enhanced chemiluminescence (Super-Signal West Pico, Pierce, Rockford, IL). Protein immunoreactive signals were scanned and the intensity of bands was semi-quantified using the NIH Imaging program.

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